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Prostate Cancer

Biochemistry, Molecular Biology and Genetics

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Preface

Prostate cancer is the most frequently diagnosed non-cutaneous malignancy in men and the second leading cause of male cancer-related mortality in the USA. The last decade has seen unprecedented progress in the detection, prognosis, treatment, and prevention of prostate cancer. These advances have been driven largely by an increased understanding of the underlying biochemistry, molecular biology, and genetics of the disease. New cell and animal models have been developed that recapitulate the natural progression of prostate cancer. New technologies have allowed scientists to view in detail the genomic, proteomic, metabolomics, and other—omic universe of cancer cells and tissues. This has resulted in a greater understanding of the pathophysiology of the disease. The purpose of this book will be to provide an up-to-date review of the biochemistry, molecular biology, and genetic changes in prostate cells that are the driving forces in the initiation and progression of cancer. It will include an overview by experts in the field of cell–cell interactions, including stem cells, reactive stromal cells, and membrane lipid rafts that are instrumental in the initiation and progression of prostate cancer. The following subjects will be reviewed:

- The role of Ets fusion gene mutations in the initiation of prostate cancer and the involvement of PTEN mutations in the progression of prostate cancer will be discussed.
- Cellular signaling mechanisms, including that of Vav3, TGF-beta, MAPK, NF-kappa-B, DAB2IP, and prostatic acid phosphatase, which are critical for the maintenance of prostate cancer cells, will be outlined.
- The role of hormone and vitamin receptors in the initiation and progression of prostate cancer, including androgen, estrogen, vitamin D, will be highlighted.
- The effect of androgen deprivation on key signaling molecules such as histone deacetylase and tyrosine kinases will be defined.
- Important cell cycle regulators such as Cyclin D will be reviewed.
- The regulation of apoptosis and autophagy in prostate cancer cells will be discussed.

Together, these reviews should give the reader a comprehensive conceptual framework of the cellular mechanism that are critical for the initiation and progression of prostate cancer. This book will distinguish itself from other books on prostate cancer, which are largely clinically oriented. Thus basic and clinical scientists, as well as students and fellows, should find this information pertinent to their fields of interests.

Rochester, MN, USA Donald J. Tindall

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Part I
Cell Biology

Chapter 1
Stem Cells in the Normal and Malignant Prostate

Norman J. Maitland

Abstract Tissues and organs like the prostate are derived from multipotent stem cells, which themselves are the products of differentiation from an original pluripotent embryonic stem cell population. Stem cells that persist into the mature prostate gland are termed tissue stem cells and are required for replenishment of the epithelial and stromal populations after damage, for example, by inflammation or gland involution after castration. While there remains some controversy over the phenotype of these cells, their ability to regenerate tissues and their inherent resistance to mutagenic and cytotoxic insults confer a unique biology on tissue stem cells. When one considers the origins of prostate cancer, the extended life span of tissue stem cells, and their ability to accumulate over time the necessary founder mutations, would imply that this primitive SC population is the cell of origin for prostate cancer. In the cancers, cells with similar primitive phenotypes are rare, but can be identified in varying proportions, depending on the markers used for isolation and the purification techniques. The tumor-initiating capacity of these cancer stem cells is many orders of magnitude higher than the majority cell population in tumors, and they display treatment resistance characteristics, which are sometimes shared with the normal tissue stem cells. The cancer stem cells in prostate cancers may therefore represent a viable target for therapeutic intervention, but there remain real challenges in the design and execution of these stem cell treatments.

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Definitions

Acute myeloid leukemia (AML) A rare cancer with the phenotype of an increase in the number of (myeloid) white blood cells in the bone marrow

Aldehyde dehydrogenase (ALDH) A member of the aldehyde dehydrogenase enzyme family, whose elevated expression levels can be used to enrich for stem-like cells

Androgen receptor (AR) Protein receptor for the male sex hormone androgen. Present at highest levels in the luminally differentiated cells in prostate

Basement membrane A complex proteinaceous boundary to each acinar unit of the prostate: It forms part of an active stem cell niche and signals to both stromal and epithelial components

Cancer cell type of origin (CCTO) The cell type within prostate from which a tumor develops

Castration-resistant Nkx3.1-expressing cells (CARN cells) A rare luminal stem cell population, which has been identified in the mouse prostate. CARN cells can give rise to both luminal and basal cells during prostate tissue regeneration induced by androgen depletion

Fluorescent-activated cell sorter (FACS) Provides a method for sorting a disaggregated heterogeneous mixture of biological cells into two or more fractions, based upon the specific light scattering and fluorescent characteristics of each cell. It is particularly useful for the identification of rare cell populations

Gleason grading A morphological classification of the abnormal prostate gland, first established by Donald Gleason (in 1966). The loss of acinar morphology is broadly predictive of patient outcome

Hedgehog, wingless (wnt) and Notch Developmental signaling pathways originally defined in *Drosophila melanogaster* which also influence embryonic prostate development and adult prostate differentiation

Hematopoietic stem cells (HSCs) Primitive cell type at the top of the hierarchy of cell types which differentiate into multiple cells types in the bloodstream and bone marrow (for example)

Induced pluripotent stem cells (iPS cells) Biologically engineered stem cells, generated by in vitro treatment of already differentiated cells (e.g., skin fibroblasts) by a cocktail of (normally) four genes, which can differentiate into multiple cell types

Mesenchymal cells (also mesenchymal stem cells, MSCs) Cells with a broadly stromal elongated morphology, which include an androgen-receptor expressing population capable of changing the behavior of the epithelial cells by signaling through the basement membrane

Orthotopic xenografts Implantation into the tissue of origin (in this case, the murine prostate)

Prostate cancer stem cells (CSCs) A generic term for the epithelial tumor-initiating cell in prostate cancer, as like a normal tissue stem cell, CSCs can differentiate into multiple cell types. Also known as tumor-initiating cells (TICs)

Prostate involution Shrinkage of the prostate gland as a consequence of castration, which is accompanied by the loss of structural acinar features

Prostate stem cells An epithelial cell, which can reconstitute all of the cells of the epithelial component of a prostatic acinus. Its basal/luminal phenotype remains controversial

Prostatic acinus (acinar morphology) The base subunit of the prostate gland, which consists of an epithelial bilayer, surrounded by an intact basement membrane and bounded by complex fibroblastic (stromal) cells. Progressively disrupted through increasing Gleason grades of cancer

Stem cell quiescence A common feature of most reserve and stem cells in tissues, quiescence implies a lack of replicative activity, in the absence of complete cellular degenerative shut down. It can be considered as an inactive slowly metabolizing cell that is primed to respond to various stimuli, including injury

Subcutaneous xenografts Describes the implantation site under the skin of the mouse host

Tumor-initiating cells (TICs) See CSCs

Urogenital sinus mesenchyme (UGM) A powerful inductive androgen responsive mesenchymal component that defines the earliest stages of prostate gland (and acinar) development in embryos

Xenografts Implantation of (in this case) human tissues into an immune-compromised mouse host

Stem Cells in Prostate Development

The human prostate is an exocrine gland with a complex anatomical structure that originates from endodermal epithelial and mesodermal stromal (mesenchymal) cells [1]. When considering the stem cells in normal and malignant prostate, it is important to take into account the development of the prostate and the signaling which results in its particular acinar morphology (see Fig. 1.1).

During early embryonic development of all vertebrates, there is a period of sexual indifference, in which the gonads of both males and females are morphologically undifferentiated. The male genital tract develops from the Wolffian ducts and the urogenital sinus (UGS) [2]. Solid epithelial buds first form as epithelial outgrowths of the UGS [3], which invade the surrounding mesenchyme. When the elongating UGS epithelial buds contact the prostate mesenchyme, there is coordinated differentiation of both the epithelial and mesenchymal components [4] followed by elongation and branching of the developing ducts to form a complex secretory network. The epithelial component is marked by fluctuating patterns of cytokeratin (CK) and androgen receptor (AR) gene expression, culminating in discrete basal and luminal cell compartments, whereas the mesenchymal cells differentiate into periductal smooth muscle and fibroblasts [5]. It is assumed, although not yet proven, that a separate stem cell component exists within the epithelial and mesenchymal compartments, from which the proliferating and interacting elements are derived.

The interdependence of the two inducing cell types was first shown (in rodent prostate) by Cunha [6] who demonstrated that UGS mesenchyme (UGM), seminal

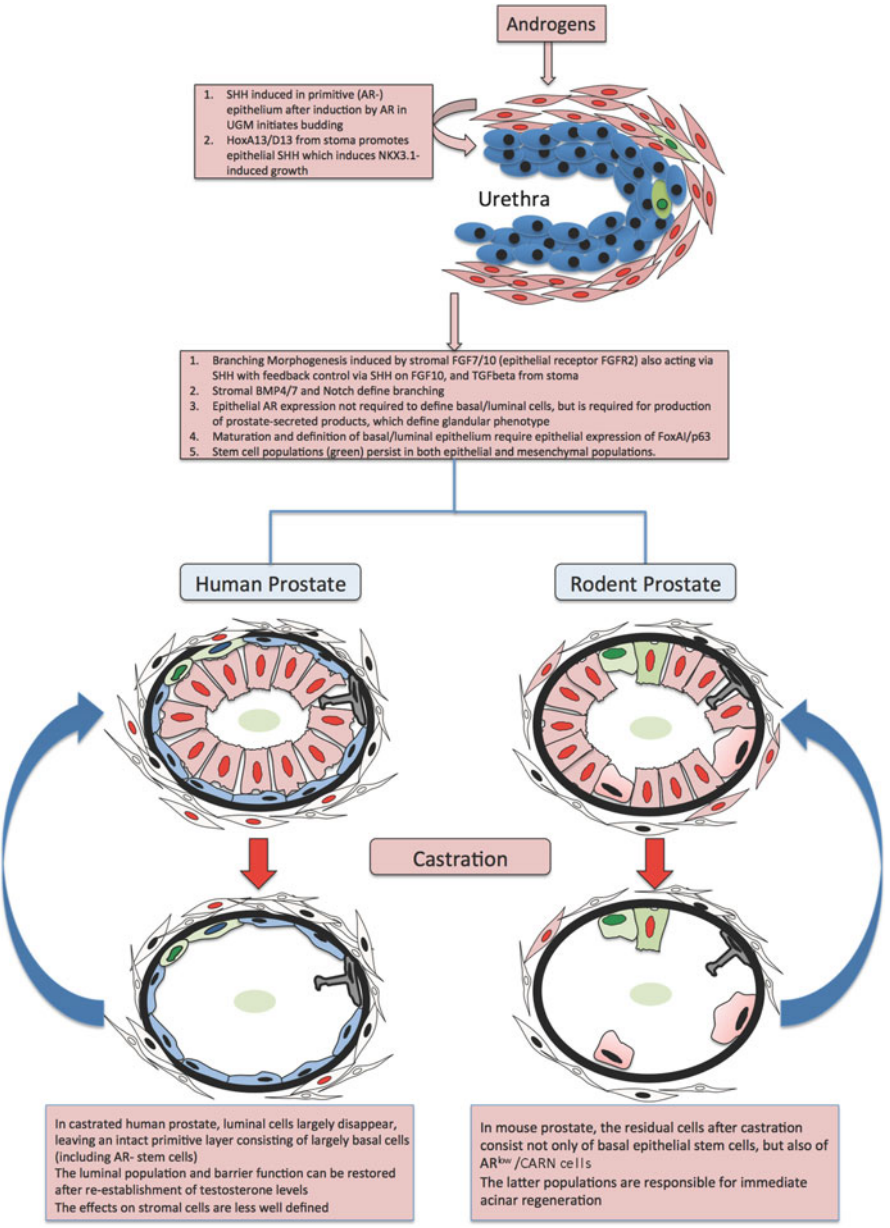

Fig. 1.1 *Stem cell localization in development of normal prostate and after castration-induced prostate gland involution*. In both embryonic and adult prostate glands, stem cells in the epithelial and stromal compartments are shown in *green*, basal cells in *blue*, and luminal cells in *pink*, with AR-expressing nuclei in *red*. Hormone-responsive stromal cells are also shown in *pink*. The influences of growth factors and hormones on epithelial proliferation and differentiation into mature glands are illustrated in the *upper part* of the figure. The key inducing effect of androgens via the stromal cells is indicated. In the *lower part* of the figure, the effects of androgen withdrawal in castration separate into human and rodent prostate, to distinguish the distinctive anatomy of the prostate from these sources

vesicle mesenchyme (SVM), UGS epithelium (UGE), and seminal vesicle epithelium (SVE) were not able to develop normally if grown alone, even in the presence of adult physiological levels of androgen. However, when the UGS compartments or the seminal vesicle compartments were cocultured, normal development of the prostate and the seminal vesicles were observed [7]. In this case it is of interest to speculate upon the definition of a stem cell in these four populations. For example, is there a common epithelial stem/progenitor for both UGE and SVE? Does this persist into adulthood, or are the cell types irrevocably defined? These stromally determined growth and differentiation/branching processes are actually continuous, and extend from late fetal life into early adulthood [2, 3], but are most pronounced during the first half of gestation [8].

Regulation of Prostate Development

While the onset of prostate development is mainly determined by the presence of androgens [9], constant exposure to the hormone is not required to initiate epithelial differentiation. For example, when UGM explants from male mice were grown in the absence of androgen, budded structures developed only when the UGM explants were obtained *after* the mice started to produce testosterone. Prior to and during bud formation, AR is initially only detected in the mesenchyme of the urogenital sinus, but is undetectable in the developing buds [1]. This was interpreted as indicating that androgens trigger an irreversible commitment, which continues in the absence of this hormone [9]. Interestingly, AR must be expressed in the UGS mesenchyme but not in the UGS epithelia, in order to promote prostatic morphogenesis, as shown by grafting of AR-deficient murine UGS epithelium in combination with (1) wild-type murine UGS mesenchyme, which resulted in androgen-dependent ductal morphogenesis or (2) AR-deficient murine UGS mesenchyme, which produced vaginal-like differentiation [10, 11].

Despite this major role of androgens in prostate biology, other hormones can regulate prostate development, some of which are detailed in Fig. 1.1. During early development, estrogen exposure modifies prostate development by altering the expression of homeobox genes such as NKX3.1 and HOX13 [12, 13]. Retinoic acid (RA) also plays an important role in prostate development mainly through the retinoic acid receptors (RARs), in the control of proliferation and differentiation in prostate epithelium [14, 15]. Mice that lack RAR gamma develop prostatic squamous metaplasia, which renders them completely sterile [16].

Hitherto unidentified molecules transmit the differentiation and proliferation-inducing AR responses from the responding mesenchymal cells to the epithelium. It is likely that these consist of positively and negatively acting independent regulators of differentiation, proliferation, and morphogenesis itself. Although still controversial, sonic hedgehog (Shh) seems to play a significant role in the regulation of branching morphogenesis [17, 18], for example, by upregulation of the transcription factor NKX3.1 [19–22] and the mesenchymal homeobox genes Hoxa10 and Hoxd13

to enhance prostatic duct formation [23, 24]. Mice with mutations or knockouts in these homeobox genes exhibit reduced size or are missing of parts of the prostate and display decreased branching morphogenesis [23–25].

Shh expression is maintained by the interaction of fibroblast growth factors FGF7 and FGF10, which bind to the epithelial FGF receptor 2 [26–29]. This process is regulated by a negative-feedback loop, as SHH is able to downregulate FGF expression [30]. Furthermore, both Activin A and Follistatin have been implicated in prostate morphogenesis [31] as well as the polysaccharide component Hyaluronan and its receptor CD44, since anti-CD44 antibodies were able to impair prostatic development [32, 33]. p63 is a key transcription factor that controls the differentiation of epithelial cells in the prostate and subsequently the smooth muscle cells, which form around the epithelium before lumen formation occurs [34, 35]. Mice lacking p63 are unable to form epithelial tissues, and Signoretti et al. [36] showed that p63 expression is essential for prostate formation, although earlier studies [34] in tissue reconstructions with p63 knockout cells had suggested that this potent regulator of epithelial progenitors was not required to generate a vestigial prostate gland.

Notch signaling can also stimulate branching morphogenesis [37, 38], but its activity is inhibited by bone morphogenetic proteins BMP4 and BMP7, which are secreted by the mesenchyme [39, 40]. TGFβ inhibits prostatic growth and decreases ductal tip number, leading to changes in the branching pattern [41, 42]. Keratinocyte growth factor has been proposed as a mediator of androgen response in rodents [43], but in the prostate, AR activity is neither essential nor sufficient for the regulation of epithelial differentiation during gland development.

Adult Stem Cells in the Normal Prostate

Human and Murine Prostates Display Different Cellular Content and Anatomical Features

Most of our knowledge of prostate development comes from studies in mouse prostate [44], but it should be remembered that mouse prostate displays distinct morphological differences from the human prostate. For example, the human prostate has a discrete glandular morphology, consisting of acini surrounded by a double layer of basal and luminal epithelial cells, whereas the murine prostate consists of four separate lobes, composed of acini bounded by a single epithelial layer [44, 45]. These differences are depicted in the lower part of Fig. 1.1.

Adult and Embryonic Stem Cells

Even in the developing embryonic mouse prostate, there is considerable evidence that the epithelial stem cells have not become restricted to a prostate epithelial cell lineage (see above). Adult tissue SCs have more limited differentiation potential

than embryonic SC [46], unless manipulated by induced pluripotent stem cell (iPS cell) techniques [47] and are committed to differentiate along more restricted lineages. Adult SCs, which generally are small, highly refractile cells with a high nuclear to cytoplasmic ratio, are present in most if not all mammalian tissues and are essential for not only tissue homeostasis but also repair and regeneration. Notably, many adult hematopoietic and solid tissue stem cells are quiescent [48–51], but are induced to proliferate after tissue insults, where the signal to break quiescence is transmitted via changes to their daughter transit-amplifying (TA) and committed basal (CB) cells. All quiescent tissue stem cells possess a high regenerative potential, giving rise to rapidly proliferating TA cells which ultimately commit to differentiation [52] into a terminally differentiated luminal cell in prostate.

Epithelial Stem Cells in the Normal Murine Prostate

If we define adult stem cells as a reservoir for tissue regeneration, which can divide asymmetrically to generate actively dividing daughter cells (transit-amplifying cells), the processes required for prostate development under UGM stimulus are apparent. In the adult prostate however, the stromal component may provide a restrictive rather than an inductive stimulus (Fig. 1.1).

The majority of epithelial cells in the adult rodent prostate depend on androgens for survival [53]. As a consequence of castration (in male rats), the prostate undergoes rapid involution and up to 90 % of the total epithelial cells are lost [53]. Whilst the remaining epithelial cells do not require androgen for survival, some of these androgen-independent cells are sensitive to androgen, as subsequent administration of exogenous androgen results in induction of proliferation and regeneration of the prostate to its original size and function [54, 55]. Cyclical induction of prostate involution and regeneration can induce >60 population doublings in the rat ventral prostate [56]. As this cycle of involution–castration can be repeated many times, a population of long-lived, androgen-independent stem cells responsible for the regeneration of the gland must therefore exist [56]. Isaacs and Coffey [57] proposed a tissue stem cell model for prostate epithelia, whereby androgen-independent stem cells give rise to a population of androgen-responsive (but independent) transit-amplifying cells. These cells should be responsive to androgens, which results in an amplification of androgen-dependent, secretory luminal cells. Even long-term castrated adult male rats (>3 years) can fully regenerate a functional prostate after androgen replacement [57].

However, this model for the prostate epithelium has not been universally accepted. For example, there is evidence that basal and luminal secretory cells can be self-replicating cell types in the prostate gland of the rat, after involution induced by castration [58, 59]. Here, in the presence of castrate levels of androgen, a population of cuboidal glandular cells persisted, in addition to the basal cells previously observed. When androgen levels were restored, both populations expanded simultaneously, but luminal cells proliferated at a higher rate compared to the basal population, implying that basal and luminal cells were both responsible for regeneration.

Ki67 antigen, which is expressed in late G1, S, G2, and M phases of the cell cycle [60], is expressed at least 100-fold more frequently within the basal cell compartment of the normal prostate [61]. However, under complete androgen blockade, luminal cells can also express Ki67 [62], a further indication that basal and luminal cell populations comprise independent and separate lineages. The point at issue is whether castration is the best method to identify tissue stem cells. If we take the immune system as an example, there are primitive repopulating cells, which can rapidly respond, and an underlying quiescent stem population, which serves as a long-term store of tissue regenerating cells [63]. Therefore, data from castration experiments in rodents do not preclude the production of luminal cells from basal cells, as the glandular cells, which persist post-castration are most likely to be the androgen-independent amplifying cells hypothesized in the stem cell model of Isaacs and Coffey [57].

The rodent remains the best animal model in which to trace epithelial cell lineages in prostate, and here the location and identity of "the" stem cells remain uncertain. Each prostatic duct consists of a proximal region attached to the urethra, an intermediate region, and a distal tip [64]. The tips of the ducts contain most of the proliferating cells and undergo growth-driven expansion when grafted subrenally in combination with embryonic urogenital sinus mesenchyme, implying that the prostatic stem cells reside in the distal region [65]. The proximal region is enriched in a subpopulation of slowly cycling epithelial cells, which possess a high in vitro proliferative potential and can reconstitute highly branched glandular ductal structures in collagen gels, i.e., implying that prostatic epithelial stem cells are concentrated in the proximal region of the ducts and give rise to the proliferating transit-amplifying cells, which can then migrate distally [66]. Both studies provide evidence that a stem cell hierarchy exists in the prostate, as luminal and basal cell components can be regenerated from proximal and distal tissue. More recently, cell surface markers have been used to more precisely identify stem cells in the murine prostate. Cell surface expression of Sca-1, or Ly6A/E: a glycosyl-phosphatidylinositol (GPI)-linked protein expressed on hematopoietic stem cells, [67] can be used to enrich for a prostate-regenerating cell population, which is concentrated in the proximal region of the prostatic duct [68]. However, sporadic Sca-1 expression has also been seen in the distal region of ducts, and therefore regenerating activity could also be attributed to Sca-1$^-$ cells.

Do Normal Rodent Prostate Stem Cells Have a Luminal or Basal Phenotype?

In support of a luminal stem cell phenotype, label-retaining cells in bromodeoxyuridine pulse-chase experiments, a common feature of many tissue stem cells, were present both in the luminal and basal epithelium and could regenerate prostate acinar structures in collagen gels. Here, the quiescent, rarely dividing (and hence

label-retaining) cells were present in both the luminal and basal epithelium [66]. The case for a more basal phenotype, equally consistent with the label-retaining experiments, was made by Burger et al. [69] who exploited the Sca-1 marker to identify potential stem cells in the proximal region of prostatic ducts, and further defined the cell type as ($CD45^{-}CD31^{-}Ter119^{-}Sca\text{-}1^{+}CD49f^{+}$) to isolate and characterize cells with self-renewal, sphere-forming, and differentiation abilities [70]: all characteristics of a potential tissue stem cell. Ultimately, Leong et al. [71] provided elegant evidence that a single cell with a largely basal phenotype ($Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$) was able to regenerate an intact prostate acinus in a renal capsule graft model. This phenotype has been further enhanced by the addition of CD166, the activated leukocyte cell adhesion molecule marker, which has been used as an indicator of poor prognosis in a number of tumors [72]. Elevated aldehyde dehydrogenase type 1 (ALDH1) expression, as originally used to identify different populations of hematopoietic stem cells (HSCs) [73], has also been exploited as a single marker to isolate similar prostate SCs [69] which also expressed Sca-1: these cells had a high proliferative potential both in vivo and in vitro. However, the levels of ALDH1 remained somewhat heterogeneous, and there is good evidence to suggest that $ALDH^{lo}$ cells mark the most primitive HSCs with the greatest developmental potential, whilst experiments with breast epithelium further imply that elevated ALDH1A3 (the most common isoform in breast and prostate) marks an early commitment to luminal differentiation [74], i.e., more in keeping with a TA/CB cell. More recently, gene expression profiling of enriched populations from both stromal and epithelial components of the tissue recombination models has emphasized the importance of established developmental signaling pathways such as Hedgehog, wnt, TGFbeta, and retinoic acid signaling [75]. Based on high keratin 5 expression, the putative (Sca-1) epithelial stem cells had a basal phenotype.

The Unanswered Questions Concerning Murine Prostate Stem Cell Identity

The intriguing questions posed by these studies are (1) whether there are indeed several tissue stem cells with different phenotypes in the mouse prostate, which could correspond to the requirements of the different lobular structures, (2) whether the several phenotypes reported by different investigators are linked by a common lineage, and (3) whether regeneration after castration, upon restoration of androgenic stimulus, is similar to surgical/damage-driven regeneration and acinar neogenesis. With regard to the latter, the straightforward explanation for regenerative capacity suggests that the tissue will regenerate from an AR-expressing cell, hence CARN or luminal precursor cells [76]. However, evidence from studies on murine prostate development also suggests that the androgen stimulus can be delivered via a stromal component (see above). To reconcile these disparate data, it is possible that the murine prostate contains a form of reserve stem cell of a more primitive and

basal phenotype, in addition to the more differentiated (CARN) cell, which can act as an "immediate early" responder to changes in physiological conditions, such as those found by differential ALDH1 expression in HSC populations [63] and in the murine colon [49]. Alternatively, classical anatomical studies of rat prostate (which is similar in its lobular structure to mouse prostate) indicated that the response of the epithelial and stromal cells in the ventral lobe to castration (apoptosis) was quite distinct from that of the more resistant dorsal and ventral lobes, which were relatively resistant [77]. Thus, the castration-resistant SC that promotes regeneration may be located in a different lobe of the prostate, whilst all of the cells in the ventral prostate may be AR^+ and castration sensitive.

Lineage Tracking Experiments in Experimental Models of Murine Prostate Development

This lineage question is one which can be resolved by elegant marking experiments such as those carried out in murine colon [78]. By employing differentiation-regulated fluorescent markers to track cell lineages in mouse prostate, Choi et al. [79] hypothesized that there should be two independent self-sustaining lineages in the murine prostate: (1) a basal stem/progenitor lineage, with no or restricted capability to differentiate into luminal cells, and (2) a separate luminal progenitor, perhaps distinct from the CARN cells, which was self-sustaining after castration. Most recently, using a complementary tamoxifen-induced lineage tracking technique, Ousset et al. [80] provided further evidence for more basal tissue stem cells, reinforcing the regeneration role of the CARN cells as a product of a basal reserve population (as discussed earlier).

Thus, even in a model organism like the mouse, in which cell fate can be traced with some certainty, the existence of a single or multiple repopulating stem cells still remains controversial.

Epithelial Stem Cells in the Normal Human Prostate

Despite the obvious anatomical and histopathological differences between the rodent and human prostate discussed earlier, and the inherent difficulties in the performance of elegant lineage tracking, there is a consistent and increasing body of evidence that human prostate tissue stem cells reside principally in the basal layer. By exploiting the heterogeneous patterns of integrin immunostaining in normal human prostate, small numbers of integrin $\alpha_2\beta_1^{hi}$-expressing cells can be seen to be randomly distributed throughout acinar and ductal regions [81]. Such cells have the useful property of rapid adherence to type I collagen, which permits their isolation based on their integrin phenotype. Expression of CD133 (as defined by the AC133 antibody), another putative stem cell antigen found in the most primitive HSCs [82],

can be used to further enrich for a primitive cell in prostate epithelia [83] where expression is restricted to the integrin $\alpha_2\beta_1^{hi}$ population. It is important to note that CD133 expression (as mRNA and protein) is more widely distributed than just SCs, and that the AC133 epitope is a glycosylation modification to the peptide backbone. Cells expressing CD133 are localized to the basal layer of the prostate, often at the base of a budding region or branching point. These cells are neither dependent upon nor responsive to androgens and do not express the androgen receptor [83]. A similar conclusion was reached by Huss et al. [84], who xenografted benign human prostate glands into immunocompromised mice and detected only $p63^+$ basal cells after extended periods of castration. These purely basal gene expression patterns were further confirmed by array-based gene expression analysis [85]. In contrast, luminal cells from normal human prostate tissues are unable to either persist in culture or initiate new prostate gland development. Prostate basal cells were also shown to have enhanced sphere-forming ability and can regenerate prostatic tissue in vivo [86], while the same basal $Trop2^+CD44^+CD49f^{hi}$ phenotype could also be induced to regenerate tubular structures containing discrete basal and luminal layers, which could be serially passaged in vivo [87]. More recently, in situ lineage tracking, which takes advantage of mitochondrial mutations accumulated in stem cells, and retained in their progeny, demonstrated that a single stem cell could regenerate an entire acinus and revealed a common clonal origin for basal, luminal, and neuroendocrine cells [88]. Lentiviral marking experiments, in which $CD133^+$ basal cells were transduced with a luminally regulated fluorescent protein gene, were also able to form vestigial prostates in vitro, and only upon differentiation (as defined by prostatic acid phosphatase expression) was the luminal fluorescent protein expressed [89].

Cancer Stem Cells in Prostate

The Origins of the Cancer Stem Cell Hypothesis

Cancer is now recognized as being very different from the original concept of a homogeneous mass of rapidly dividing cells: indeed most if not all tumors are heterogeneous with respect to (1) their potential for self-renewal, (2) the ability to reconstitute tumors upon transplantation [90–92], and (3) rapid proliferation (content of dividing and dying cells). These transplantation experiments produced a hypothesis, which proposed that cancers arise from a rare population of *cancer stem cells*. To confirm this hierarchical CSC model for the initiation of cancer, it was necessary to purify distinct populations of cells (normally based on cell surface phenotypes) within tumors and to determine their tumor-initiating properties. This was first reported in studies of acute myeloid leukemia (AML) [93] where it was shown that the $CD34^+$ cell fraction from a number of human patients contained leukemia-initiating cells. Thus, AML is organized as a hierarchy, in which only a rare subset of cancer cells

Table 1.1 Cancer stem cell characteristics

CSC property	In vitro	In vivo	Stem cell characteristics	Cancer characteristics
Can reconstitute the original tumor in an immuno-competent murine host	✗	✓	✓	✓
Shows relative resistance to chemo- and radiotherapies	✓	✓	✓	✓
Is responsible for tissue/tumor regeneration after injury	✓	✓	✓	✓
Can divide for the lifetime of the tumor/host organism	✗	✓	✓	✓
Divides asymmetrically	✓	✓	✓	✗
Constitutes a small fraction of the tissue/tumor cell content	✓	✓	✓	✓
Are either quiescent of slowly proliferating	✓	✓	✓	✗
Can differentiate to produce a lineage of other differentiated cell types	✗	✓	✓	✗
Can maintain its population independently of input from other cell populations	✓	✓	✓	✓
Matrix invasion: metastasis	✓	✓	✗	✓

possess the ability to initiate new tumor growth and can recapitulate the original tumor heterogeneity. Similar results have been obtained with subpopulations of tumor cells from breast [94], brain [95, 96], pancreas [97], liver [98], colon [99], lung [100], and endometrium [101], as well as the prostate [102], where cells with CSC characteristics (Table 1.1) have been identified. One potential confounding factor for the hypothesis was that the markers used to identify the CSCs were identical to those that could be used to identify normal tissue stem/progenitor cells. This could be interpreted either (1) that there was a contamination of normal stem cells within a tumor (since no cellular purification, even by sequential immunomagnetic or FACS selection, is more than 98 % effective) or (2) that CSCs and their normal counterparts share many phenotypic markers, implying a stem cell origin for the cancer stem cells (see below). Thus, the first goal in cancer stem cell studies in prostate was to identify the cancer cell type of origin (CCTO). There remains an important question regarding the phenotype of the CCTO: does hormone-responsive prostate cancer, which is predominantly luminal, develop from a luminal cell in the normal prostate, or do the initiating (mutational) events occur in a basal cell, which can differentiate, perhaps aberrantly to produce a replicating luminal tumor? To some extent therefore, the study of tumor initiation becomes an analysis of normal and aberrant prostate epithelial differentiation, as defined by a series of putative cell-type specific markers.

Whilst some conclusions can be made from analysis of fresh human tumors (see below), there is a greater precedent for the study of tumor initiation and cell lineages in murine models of cancer, for example, in colon cancer [103]. In this respect, a number of such models have been exploited in attempts to define the CCTO in prostate cancer.

Defining a CCTO in the Mouse Prostate

Probably one of the best natural models of tumor initiation in mice is a conditional PTEN deletion mouse model [104] where prostate-specific homozygous deletion of PTEN by cre-recombinase is driven by the ARR2 probasin promoter. Further studies with this mouse found an expansion of $p63^+$ basal cells, which share expression of the Sca1 and BCL-2 genes that are also found in stem/progenitor populations [105]. Interestingly, the probasin promoter appeared to be active in both basal and luminal cells, proportions of which both express the androgen receptor (normally restricted to luminal cells in human tissues). The same rat probasin promoter has been used for the development of TRAMP mice [106] or SV-40 T-antigen (TAg) rats [107], and its androgen inducibility used as evidence that CaP arises primarily from AR^+ luminal cells. In another PTEN knockout murine model of CaP, using a similar androgen-regulated human PSA-promoter, Ma et al. [108] identified luminal progenitor cells that are able to act as TICs. Moreover, Korsten et al. [109] showed in the same model that the genetic alterations are first seen in a subset of luminal cells, which express Trop2 and Sca-1, providing further evidence that the luminal cells are the cell of origin.

In the probasin-driven PTEN-null mice, Liao et al. [110] had previously shown that prostate CSCs enriched on the basis of a $Lin^-Sca\text{-}1^+CD49f^{hi}$ phenotype had a strong capacity to form tumor-like spheroids in vitro and grafts in vivo, and that introduction of a series of genetic alterations (resulting in increased AKT, ERG, and AR signaling) into $Lin^-Sca\text{-}1^+CD49f^{hi}$ cells from the basal ($p63^+$) fraction of normal murine prostate produced luminal-like disease, characteristic of human CaP upon transplantation into immunodeficient mice [111]. Importantly, subsequent studies also revealed the influence of the stromal tumor microenvironment, since cancer-associated fibroblasts supported and potentiated the stemness and growth properties of the CSCs [112]. Other studies suggest that the disease is derived from intermediate progenitors that have acquired the ability to self-renew. For example, Xin et al. [68] showed that introduction of constitutively active AKT—a surrogate for PTEN loss—into Sca-1-enriched murine prostate epithelial cells (which were responsible for regeneration of normal murine prostate and had evidence of both basal and luminal lineages) resulted in the initiation of prostate tumorigenesis.

In another transgenic model of prostate, Wang et al. [76] showed that murine CARNs (castration-resistant Nkx3.1-expressing cells) could also self-renew in vivo and reconstitute vestigial prostate ducts in renal grafts using single-cell transplantation assays. Furthermore, upon deletion of the PTEN tumor suppressor gene in CARNs, carcinomas were rapidly formed, together with androgen-mediated prostate regeneration [76].

The development of precise genetic marking technology, using cells conditionally marked by fluorescent proteins under the control of differentiation/lineage-specific promoter sequences, has introduced a new layer of complexity onto a number of murine cancer models [113]. As originally exploited in breast cancer, this technology is designed to overcome concerns about the validity of

transplantation experiments as a true model for TICs and the lack of cell-type specificity in both the Probasin and NKX3.1 promoters used to drive gene knockouts [114, 115]. In prostate, Choi et al. [79] recently showed that the basal and luminal cell lineages were separable in terms of initiating cells or stem cells, and furthermore that luminal cells were more sensitive to tumor initiation by PTEN knockout than basal cells, which could only result in cancer after differentiation into a luminal cell (and with a longer latency). While the ultimate TIC (if it exists) in mouse systems was not identified by these elegant studies, we are left with the major conclusion that the deregulation of the exquisite control of cell numbers and differentiation required in a normal prostate is a critical part of tumor initiation, perhaps more than, or as a precursor to induction of proliferation.

Can We Extrapolate Murine CCTO Studies to Human Prostate?

Although murine models mimic the development of and progression of the human disease, they do not necessarily represent a valid model for the identification of the CCTO in human CaP. It has been assumed that prostate cancer arises from the terminally differentiated luminal cells, because the bulk population of tumor cells in the most common form of prostate cancer expresses luminal cell-specific markers (cytokeratins 8, 18 AR, PSA, and PAP), but lacks expression of basal cell markers, such as Ck5, 14, and p63. Some time ago, in early fractionation studies, Liu and coworkers [116] observed that most primary tumors consist of ($CD57^+$) luminal cells, whereas the majority of metastases are enriched for cells with a more basal phenotype ($CD44^+$) and that the luminal phenotype was regenerated by coculture with prostate fibroblasts. Conversion from $CD57^+$ to $CD44^+$ was rarely if ever observed.

In human cells and tissues, there is also a strong body of evidence supporting the basal cell origin of prostate cancer. Using the same antigenic markers that identified normal basal SCs, putative basal CSCs have been isolated in our laboratory from human CaP biopsies with a $CD44^+$ integrin $Ð_2β_1{}^{high}CD133^+$ phenotype [102]. Only this primitive cell population was able to self-renew in vitro. Moreover, under differentiating conditions, $AR^+PAP^+CK18^+$ luminal cells could be identified in these cultures, suggesting that they were derived from the more primitive population.

CCTO Cells in Human Prostate Cancer

Unlike the murine studies, precise lineage tracking for human normal cells and their transformation into cancer are currently impossible to carry out. However, for many years, overexpression of the SV-40 TAg (which results in suppression of the tumor suppressor genes p53 and RB [117], and the protein phosphatase 2A (PP2A) gene [118]; causing loss of cell cycle control, genomic instability, and enhanced proliferation) has been used to extend the lifespan of human prostate epithelial cells. These effects are sufficient to immortalize benign human prostate cells in vitro [119, 120]. The targets for the SV40 TAg are invariably transit-amplifying cells of primary

prostate epithelium, which proliferate strongly in culture, but for a limited time only. This proliferation and the immortalization achieved by TAg are independent of AR expression. In these early experiments, no effects were seen when enriched luminal cells from normal and benign primary prostates (which survive for short periods in cell culture and can be transfected with indicator genes) were transfected with Tag constructs. These luminal cells are almost exclusively quiescent/senescent and represent a terminally differentiated cell population, which fails to respond to androgens, except for the expression of luminally defined genes such as PSA.

In human cells, the correct microenvironmental conditions, i.e., addition of "activated" cancer-associated fibroblasts, were required to induce tumors in mice after extended passage of BPH1 cells (which retain expression of the immortalizing SV40 Tag) in vitro [121]. This is the human equivalent of the TRAMP model and argues strongly for the vital role of cellular interactions in prostate carcinogenesis and differentiation [122].

Again, similar to murine studies, $CD49f^{hi}Trop2^{hi}$ cells from the basal fraction (but not the luminal fraction) of human primary prostate tissue, transfected with expression vectors to increase AKT, ERG, and AR signaling, recapitulated the histological and molecular features of human CaP upon transplantation into immune-deficient mice [123]. Similar reactivity was seen with cells selected on the basis of elevated expression of the CD166 IgG family cell adhesion molecule [72].

Identification of Cancer/Tumor-Initiating Cells

We have attempted to distinguish two frequently confused terms. In the previous section, the term CCTO was used to describe the cell type in which the changes leading to a prostate cancer first arise. Given the age profile of most prostate cancer patients, the emergence of a cell capable of existence as a free-standing cancer might be expected to take up to 20 years to generate. Some doubt remains about the time of origin of human prostate cancers. For example, whilst the first diagnostic signs of prostate cancers based on elevated plasma PSA are seen in late 30s to early 40s in men [124], the progression to achieve this marker level: accumulation of mutations and increased angiogenesis producing higher plasma levels of PSA, must be considerably longer. Colon cancer neogenesis has been mathematically related to increase in tissue volume during adolescence [125], and cervical carcinomas arise in the adolescent unstable epithelial boundary within the cervix—the transition zone—although probably as a result of a viral infection of the susceptible epithelium [126]. The initiating events in human prostate cancer most likely occur at the time of most rapid tissue expansion in the prostate, i.e., during the massive androgen-driven tissue generation at puberty [127]. Such tissue expansion is precisely the time when mutations can arise and be propagated, as it is likely that the stem cell pool is also expanded at this time. If such mutations are random, then only those with a selective advantage will be maintained in an actively replicating cell. However, the stem cell compartment is quite distinctive: in adult life, tissue stem cells replicate more rarely and probably in response to tissue damage.

There have been many proposed initiators for human prostate cancer such as environmental chemicals, viral infections, etc. (review in Key [128]), but perhaps the most convincing evidence has been provided with respect to persistent infections in prostate, resulting in cycles of inflammatory response/epithelial tissue damage [129]. We have recently reviewed the evidence for this in a stem cell context [130]. Despite residual uncertainty over the nature of the stem cell in normal prostate, there is good evidence to suggest that the CCTO resides in the stem cell compartment, either as the tissue stem cell itself, or as the immediate progeny of the SC: the transit-amplifying cell.

In this regard, one of the most significant gene ontology terms in the phenotype of prostate CSCs (see below), relative to benign equivalents, is "response to inflammation" [85]. We have hypothesized that, after repeated rounds of inflammatory stimulation, an "addiction" develops to proinflammatory cytokines such as IL6, the receptors for which are expressed in the normal tissue stem cells, resulting in the establishment of an autocrine loop, in which the CSCs now express elevated levels of the cytokine. One outcome of this is elevated NFkB signaling [85, 131, 132], which has been linked to more malignant behavior in prostate cancers in general [133].

Cancer-Initiating Cells from Human Prostate Cancer Tissues

It is likely that prostate CSCs will share many properties and phenotypic markers with normal tissue SCs, independent of origin, given the evidence from other tissue systems, e.g., leukemias [93], and solid tumors such as breast [94]. On this basis, we and others set out to fractionate biopsied primary human tissues from prostate cancer patients in order to enrich for the tumor-initiating cells (TICs) within a tumor mass. For a heterogeneous tumor such as prostate, where there are major contaminants of both stromal cells and normal epithelium in biopsies, such "purifications" are likely to represent an enrichment at best, although the use of metastatic tumor material, selected for prostate markers, provides a better source of homogeneous tumor. With this strategy, using expression of the markers CD44 (to enrich basal cell populations), rapid adhesion to collagen I matrices (to enrich for cells expressing high levels of $\alpha_2\beta_1$ integrin), and the particular form of the glycosylated "stem cell" marker CD133 (recognized by the AC133 monoclonal antibody), we were able to enrich a population of "CDCs" [102]. These cells had properties (see Table 1.1) strongly suggesting that they were the elusive CSCs, which constituted approximately 1 in 1,000 of the tumor mass. Similar cells were subsequently isolated with the same properties, both by FACS sorting for antigen expression [131] and by a modified Hoechst 33342 dye effluxing assay [134].

Table 1.2 Selection markers for human prostate cancer stem cells

Cell line/*xenograft*	CSC selection phenotype	References
PC3	$Fam65B^{High}/Mfl2^{low}/LEF1^{low}$	[201]
PC3-MM2	$CD133^+/CD44^+/CD166^+$	[162]
PC3	$CD133^+/CD44^+$	[202]
PC3M Pro4luc	ALDH/integrin αv	[142, 203]
DU145	$CD133^+/\alpha2\beta1$ integrin$^+/CD44^+$	[204]
LNCaP	$CD44^+/CD24^-$	[143]
hTERT-immortalized RC165N and RC-92a	$CD133^+$/CXCR4	[205]
CWR22	TRA-1-60$^+$/CD151/CD166	[131]
LAPC-9	Hoechst 33342 effluxing side population	[187]
BM18	ALDH1A1, NANOG	[206]
Multiple	$CD44^+$	[144]
DU145, *LAPC4*, *LAPC9*	Integrin α2β1High/$CD44^+$	[207]
22RV1	$CD117^+/ABCG2^+$	[208]
22RV1	ALDH1/	[161]

Note: Experiments with human xenografts in immunocompromised mice are shown in italics

Cancer-Initiating Cells in Human Prostate Cancer Cell Lines

Given the difficulties in isolation of pure cell populations from prostate tissues, it is not surprising that many investigators have exploited a variety of prostate cancer cell lines and xenografts to provide evidence for tumor-initiating cell populations, which are phenotypically and behaviorally distinct from the previously assumed homogeneous cell cultures in current use. Some of these experiments, and the variety of phenotypes exploited, are listed in Table 1.2.

A remaining controversy surrounds the use of the CD133 marker as a selective tool for CSCs from tissues and cell lines. While the AC133 epitope of CD133 (a glycosylation epitope) has successfully been used in a number of studies, e.g., DU145, RC-92a, and variants of PC3 in some laboratories (Table 1.2), others have not been able to reproduce these data [135–137]. This variability could reflect either known heterogeneity in strains of cell lines or perhaps more likely the variable and rather low and microenvironment modulated expression levels of the AC133 epitope (in contrast to the more universally expressed CD133 peptide backbone) as discussed recently by Pellacani et al. [138].

Similarly, high expression of aldehyde dehydrogenase ($ALDH^{Hi}$) has been used as a marker for TICs in a number of cancers (by the *Aldefluor* assay), such as multiple myeloma and breast carcinomas [139, 140], but also stem-like or perhaps progenitor cells in CaP cell lines [141]. A subpopulation of human CaP PC-3 M cells, with high ALDH activity ($ALDH^{Hi}$ expressing the integrins $\alpha_2\beta_1^+/\alpha_6\beta_1^+/\alpha_v\beta_1^+$ and $CD44^+$), showed both enhanced clonogenicity and invasiveness in vitro and enhanced

tumorigenicity and increased metastatic ability in vivo [142]. In the case of ALDH, the precise gene that renders the CSCs reactive in the Aldefluor assay also remains controversial. In an in vitro LNCaP model, $CD44^{+}CD24^{-}$ basal prostate stem-like cells formed colonies in soft agar and developed tumors in NOD/SCID mice when as few as 100 cells were injected into immunodeficient mice [143]. In addition, a $CD44^{+}$ population from xenograft tumors and cell lines had enhanced proliferative potential and tumor-initiating ability in vivo compared to $CD44^{-}$ cells [144].

Rajasekhar et al. [131] have shown that a small population of TRA-1-60^{+} $CD151^{+}$ $CD166^{+}$ cells, isolated from established human prostate xenograft tumors, exhibit stem-like cell characteristics and recapitulate the cellular hierarchy of the original tumor in serial xeno-transplantation experiments. Moreover, the TRA-1-60^{+} $CD151^{+}$ $CD166^{+}$ cells expressed basal cell markers and showed increased NF-kB signaling. Significantly, the cells did not express the AR; this result agrees with our own data showing a lack of AR expression in basal prostate SCs [102, 145]. In addition, we had previously shown that NF-kB expression is increased in $CD44^{+}\alpha_2\beta_1^{high}CD133^{+}$ cells isolated from human CaP tumors [85] and that NF-kB inhibitors, such as parthenolide, abolished colony-forming activity: a key property of CSCs.

A basal SC-enriched prostate epithelial side population (SP) has also been isolated from human prostate tissues, utilizing a modified Hoechst 33342 dye efflux assay [134]. 3D culture of the SP cells led to the production of spheroids, which had acinus-like morphology and expressed basal cell markers, i.e. they displayed a differentiation hierarchy.

Tumor-Initiating Cells in Primary Human Prostate Cancers: Biological Properties

1. **Tumor initiation in vivo:** the "gold standard" for identification of cancer stem cells is the ability to successfully initiate a xenograft tumor, which displays phenotypic characteristics similar to those observed in the original human cancer in immune-compromised mice [146]. However, this assay remains controversial, as shown by experiments with human melanomas, where multiple cell types were capable of initiating tumor growth [147]. However, a more precise definition of phenotype can overcome some of the difficulties with this assay [148]. Additionally, the strain of immune-compromised mouse also seems to influence the "take-rate" of such xenografts. For example, in our own studies, tumor initiation with primary tissue grafts initiated from human prostate tumor biopsies in a Natural Killer—B and T $cell^{-}$ host (the $rag2^{-/-}$ gamma $C^{-/-}$ mouse) was tenfold better than the take rate in the original and commonly used nude (nu/nu) athymic mouse. As discussed previously, in prostate tumors, the phenotype is influenced by the presence of an activated stromal component, but this is rarely apparent in such murine models, where a rapid invasion of mouse stromal cells is invariably observed. More recently, a further complication to these models has arisen, due to the observed transfer of murine retroviruses from the mouse tissues into the human tumor cells, conferring a growth advantage, which means that the infected

cells compete out the original human tumor material. Since some of these xenografted tumors also actively produce the retroviruses, the potential for cellular cross-contamination is significant, as shown by the isolation (and transmission) of the XMRV from 22RV1 [149]. These retroviral effects seem to be restricted to longer established tumors after considerable numbers of passage in murine hosts.

2. **Cell colony initiation in vitro:** however, the core property of the CSCs, vis-a-vis the ability to *initiate* new tumor growth, from a small number of cells (in contrast to the 10^5–10^6 normally used in tumorigenicity studies), remains the most fundamental property of a CSC. This ability is often equated with the ability to initiate single-cell colony growth in two or three dimensions in cell cultures. This latter assay is often however misinterpreted on two grounds. Rapid colony or spheroid (prostatospheres in 3D) formation [150] can be the result of aggregation rather than asymmetric division and growth. Therefore, it is important to consider cell cycle times when carrying out such experiments. First, a 32-cell colony (the product of five population doublings) is unlikely to form within 2–3 days, and is more likely after 10–14 days. Second, both the SC and their daughters can initiate colony formation in 2D, but only true stem cells are able to initiate secondary colony growth: a more complex and time-consuming study, which is frequently omitted. Recently, Chen et al. [151] cast further doubt on the veracity of the prostatosphere assay for tumor-initiating cells from primary tissues.
3. **Matrix invasion:** if cancer stem cells are indeed the metastatic cell type then they should also display an increased invasive capacity in assays such as the modified Boyden chamber, compared to normal cells. In fact, Collins et al. [102] showed that only CSCs from high Gleason grade prostate cancers were 2–3 times more invasive than the most invasive prostate cancer cell lines in this assay.

Tumor-Initiating Cells in Primary Human Prostate Cancers: Gene Expression Patterns

The phenotype of tumor-initiating cells remains quite variable for both prostate cell lines and tissues. Just why such a lack of consensus exists is unknown at present. However there are a few possible explanations. Cells identified on the basis of a single-cell surface marker are likely to still be heterogeneous, particularly from tissues, but equally (and perhaps unexpectedly) from established cell lines. Thus, any overall phenotypic analysis is likely to reflect either the majority population or that of the most highly expressed genes. In prostate, the most highly expressed genes are those induced by luminal differentiation [85, 152], whose expression is elevated by up to 10^5-fold compared to the same gene in primitive undifferentiated cells. Accordingly, there have been relatively few reports of complete genomic profiling of gene expression in prostate, compared to leukemic stem cells for example. Such studies are vital to determining actual phenotypes and in the identification of mechanisms for the generation of the cancer stem cells, which may have left an imprint in the CSCs, but perhaps more importantly for the development of truly CSC-specific therapies.

For many determinations, the focus of attention has been in commonality of gene expression between embryonic stem cells, e.g. genes associated with self-renewal, and the prostate CSCs. Gene families such as wnt and hedgehog and individual ESC genes such as Bmi1 have all been identified [153, 154]. Unbiased profiling of purified populations (see above) has been carried out on both primary cultures [85] and tumor xenografts [131]. While upregulation of various individual genes was demonstrated, a strong common theme from these studies was the activation of genes characterized in the Gene Ontology as those that respond to inflammation. This correlates well with proposals that persistent inflammation from prostatitis for example is related to prostate cancer development [130, 155]. More recently, Pascal et al. [156] fractionated a number of xenografts, tissues, and cell lines according to their ABCG2/CD44 ("progenitor cell") expression and compared total gene expression patterns to those in embryonal/germ cell tumors and an embryonic stem cell line. In these elegant comparisons, the problems associated with minor and major cell populations and indeed heterogeneity were emphasized. A more restricted phenotyping was reported in $CD44^+$ cells from DU145 and LAPC4 and nine xenografts by Liu et al. who focused on small untranslated RNA expression [157]. Whilst there was considerable heterogeneity in miRNA expression between subfractions of $CD44^+$ cells (side population, $CD133^+$, and $\alpha2\beta1$ integrin overexpression), subfractions of these cells from cancers frequently downregulated microRNAs with a tumor-suppressive nature such as miR34a and Let7b/a, which have a functional role in tumorigenicity. It is now clear that even small variations in miRNA expression can affect gene expression observed in different cell subpopulations during epithelial cell differentiation in the stem cell compartment.

A number of similar whole genome expression analyses have been carried out on subfractions of established in vitro cancer cultures such as DU145 [158–160], 22RV1 [161], and PC3 [162, 163] cells. In general, these studies have identified the presence of a stem-like component within the tumors which could be enriched under hypoxic conditions [164] and by treatment with hepatocyte growth factor [159]. They provide systems for mechanistic testing, which must be related to the situation in primary and castration-resistant tumors in human, while revealing cellular heterogeneity and the significance of minor cell populations even in these long-established cell lines.

Asymmetry and Control of Gene Expression in the Normal and Malignant Prostate Tissue Stem Cell Compartments

The presence of chromosomal rearrangements and mutations provides an ideal tool for lineage studies in tumor biology. The most common genomic alteration in CaP is the generation of fusion genes, which place the ERG oncogene under the control of a strongly prostate-specific androgen-regulated gene TMPRSS2 [165]. This fusion is detected in about 50 % of human prostate tumors [166]. Identification of

the TMPRSS2–ETS fusion genes in ~20 % of PIN lesions suggests that it is an early event in prostate tumorigenesis [167]. We recently showed that TMPRSS2–ERG is expressed in CD44$^+$ integrin $\alpha_2\beta_1^{high}$CD133$^+$ cells from primary tumors [85] in support of the hypothesis that the cell of origin of CaP is a basal SC [145]. However, the expression pattern most frequently observed, when allied to the presence of the fusion [168], confirmed changes in the expressed allele of TMPRSS2 between the stem cell populations and the daughter cells. In TMPRSS2–ERG + tumors, the fusion was expressed in the CSC population, but not in the products of the (presumed) asymmetric division, which produces transit-amplifying cells, where the unfused TMPRSS2 allele was expressed. This was surprising, since expression of the fusion gene is androgen regulated, yet CD44$^+\alpha2\beta1^{high}$CD133$^+$ cells are AR$^-$. However, other transcription factors, in addition to AR, for example, the estrogen receptor [169], could play an equally important role in the regulation of TMPRSS2–ETS expression, since AR-mediated upregulation of transcription, even in model systems, is consistently less than tenfold, which cannot account for the high levels of TMPRSS2 expression seen in many tissues (some of which do not express the AR). In the majority differentiated cells in tumor-derived material, TMPRSS2–ERG expression again became randomized, and at higher levels, as AR expression increased. Similar evidence of asymmetric division was also observed by Qin et al. [170], on the basis of asymmetric segregation of protein in the LNCaP and LAPC9 cell lines, where the PSA-/^{low} tumor-propagating cells divide to produce an AR + PSA^{high} daughter. The gross changes in gene expression seen in this asymmetry are again an indication of global changes in gene expression characteristic of potent epigenetic control [171] such as that achievable by miRNA [172].

Cancer Stem Cells as Minimal Residual Disease and the Origins of Therapy Resistance

In prostate, the phenotype provides strong evidence for CSCs as a source of preexisting therapy resistance within a tumor mass (Fig. 1.2). Similar mechanisms of resistance have been noted in CSC fractions from other major tumor types in human (Table 1.3). Most prostate cancers are highly dependent on androgens for growth and survival [173] upon initial diagnosis, a seminal finding that has prompted the use of increasingly sophisticated androgen deprivation therapy (ADT) to treat both locally advanced and metastatic CaP (reviewed by Denmeade and Isaacs [174]). However, most patients invariably develop castrate-resistant prostate cancer (CRPC) [174], with multiple nodes of metastatic disease, which is incurable by current treatment regimes. There is evidence from TRAMP mouse studies [175] that castration can *select* for this more aggressive and metastatic disease. In the LAPC9 human xenograft, a fluctuation analysis of CRPC development indicated that resistance probably results from a clonal expansion of rare androgen-independent cells (1 per 10^5–10^6 androgen-dependent cells) [176]

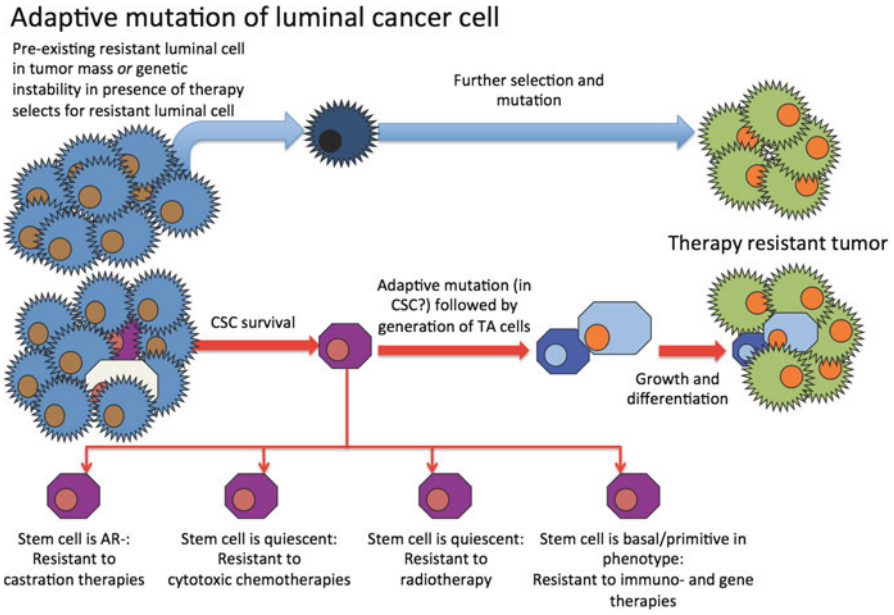

Fig. 1.2 *Development of treatment resistance in human prostate cancer*. The two hypothetical sources of a resistant tumor (*green*) are shown. In the *upper track*, a resistant tumor derives from adaptive mutation of a susceptible cell with a luminal phenotype (*blue*) from the tumor mass (stochastic change, as a result of DNA instability in the tumor cells). In the *lower panel*, the source of the resistance-initiating cell is a distinctive CSC (*purple*) with a primitive basal phenotype, which can subsequently develop the new phenotype required to exist in the post-therapy environment (e.g., low androgens or high anti-androgens after castration). The target for this adaptive mutation, i.e., a CSC or a progenitor/transit-amplifying cell (*pale blue*), is not known

Table 1.3 Some mechanisms of resistance to common treatments attributable to cancer stem cells

Resistance mechanism	References
Microenvironment/Niche	[209]
Multidrug resistance/drug efflux	[210, 211]
Detoxifying enzymes	[212]
microRNAs	[213, 214]
Cell cycle checkpoints	[215]
DNA repair	[216]
Inhibition of apoptosis	[217]
Stem cell maintenance	[182]

rather than a reversion mutation. This cell has the correct frequency for a CSC as defined in the original studies of [102], but the alternative luminal cell dedifferentiation to a more stem-like property can still not be excluded. In LNCaP and LAPC9 cells, Qin et al. [170] were able to demonstrate the presence of an appropriate number of pre-existing PSA-/^{low} cells, in these AR^{+}/PSA^{high} cell lines, in

agreement with the pre-existing resistance mechanism. Castration-resistant cells frequently show an increased AR expression [177], mutations in the AR gene [178], alterations in AR corepressor–coactivator function [179], or even genomic amplification of the AR locus [180], but the presence of such an amplification has not yet been determined in the SC population, and AR expression at the protein level is either absent or below detection levels [145]. Since mutations would "fix" a stem cell in a particular relationship with its microenvironment, and would restrict its ability to respond to restoration of pre-castrate androgen levels, epigenetic control is more adaptable and likely. The expression of AR in a SC population in humans and the basal/luminal nature of the TIC has recently been reviewed [181].

The other forms of primary treatment for human prostate cancers at earlier stages (apart from surgery) are either external beam or brachytherapy-based radiotherapies. Given the lifetime need for stem cells in normal tissues, it makes sound sense for SC to exhibit either a resistance to radiation-induced damage, for example, by manipulation of reactive oxygen species [182], or an enhanced capacity to repair such damage (or indeed both). These properties have been observed in other tissue types [183]. If we presume that the SC is indeed quiescent, and is located in a site of relatively low oxygen tension (hypoxia), then there are a number of mechanisms to explain such a resistance [184, 185]. Application of hypoxic conditions to prostate cancer cell lines is sufficient in some cases to induce a stem-like phenotype [164]. The theoretical basis for radiotherapy of tumors has always been their rapid cell cycle times, but for a quiescent prostate CSC there will be inherent resistance. Our experiments with primary cultures from human tumors (F Frame, manuscript submitted for publication) have confirmed the quiescence hypothesis and also detected a characteristic form of chromatin packing in prostate CSCs (but also in normal tissue SC) which mediates this effect.

Standard chemotherapies targeting cycling cells have been remarkably inefficient in the treatment of advanced stage prostate cancer, after failure of hormonal therapies (i.e., CRPC). If we again consider the quiescent and hypoxic nature of SC, this does not seem surprising in retrospect [186]. However, the process can also be active: enhanced repair and chromosomal packing can also restrict access of bulky DNA damaging agents. The presence of CSCs in a "side population" expressing ABC transporters [134, 187] could also enhance the efflux of chemotherapeutics, although the pattern of expression (S Klein et al. unpublished and [85]) implies that efflux cannot be the only such resistance mechanism. In drug-tolerant DU145 cells, which were surprisingly less tumorigenic than the parental population, a stem-like population ($CD44^+$, expressing for example higher levels of ALDH1 and BCL2) was detected. Yan et al. [188] concluded that these changes were more likely to be epigenetic, as reversion was possible. Such epigenetic changes would be a "smart" strategy for stem cells (not just CSCs), reacting to environmental changes, in contrast to irreversible mutations, as discussed earlier for AR expression.

Strategies to Develop Cancer Stem Cell Therapies for Prostate Cancer

New In Vitro and In Vivo Testing Systems for Prostate Cancer Stem Cell Therapies

Whilst the phenotypic nature of prostate CSCs remains somewhat controversial, the evidence in favor of their existence in almost every major tumor type, including prostate cancers, is now quite convincing [103, 113]. The major questions posed regarding their existence are (1) exactly how can we target them for destruction, (2) given that they must exhibit resistance, how can we overcome that resistance, and (3) can the established cancer drug development paradigms be used for such studies?

To address the last question, it is becoming clear that the CSC fraction of a tumor does not possess the rapid proliferative properties of the tumor bulk. Indeed they appear to be largely quiescent in prostate, expressing low or undetectable levels of Ki67 and PCNA (F Frame, manuscript in preparation, [189–191]). Cancer SCs are also extremely rare in tissues, but less so in some cell lines (stem-like rather than true SC). Therefore, assays describing the effects of CSC therapeutics on tumor or cell growth (shown in Table 1.4) are probably targeting another cell type (but perhaps the stem cell simultaneously). It is even likely that anti-CSC agents already exist, but would have been rejected in high-throughput screening of anti-proliferative agents, as carried out by the Pharmaceutical industry. The ideal drug (or other agent such as therapeutic antibody) should indeed target both the CSC and the bulk cells of the tumor, but given the distinct phenotypic and behavioral differences (e.g., [85]), this is unlikely to be found. However, a number of agents have been identified recently, but these have only rarely been applied to prostate cells and tissues (Table 1.4). Note that some of these CSC therapeutics were even selected for their ability to induce growth arrest and tumor shrinkage i.e. incompatible with CSCs as the primary and sole target.

As summarized in Table 1.2, a closer consideration of the unique properties of CSCs is more likely to yield the required assays, but the scale of the screens will be limited by the yield of CSCs, although there have been strategies proposed to expand these, mainly again from cell lines.

Targeting Susceptible Genes and Signaling Pathways in Prostate Cancer Stem Cells

Application of the burgeoning knowledge of the biology and phenotype of CSCs from prostate has however revealed a number of feasible therapeutic approaches (as shown in Fig. 1.3 and Table 1.5). First, direct killing of the CSC's is possible by application of a CSC-specific apoptosis-inducing therapeutic (Fig. 1.3a). In this

Table 1.4 Cancer stem cell treatments in development

Agent	Mode of action	Read-out of activity	Source of CSC	References
Anti-DLL4 MAb	Notch signaling (ligand), angiogenesis	Loss of TIC (CL[a])	Colon (breast)	[218]
Metformin	miRNA/mTOR (diabetes)	Proliferation, migration, and invasion (CL)	Pancreas and breast	[219]
Salinomycin	K^+ ionophores	Toxicity in CL	Breast	[220]
MiR34a siRNA	CD44 inhibition	Tumor induction and metastasis (xenograft CL)	Colon, breast, prostate, and lymphoma	[163, 221–223]
Sulforaphane	Natural product: HDAC and GSTP1, Wnt signaling	Cancer growth (CL)	Breast, pancreas, and prostate	[224–227]
Curcumin	STAT3 signaling, growth factor receptors, and MiRNA	Cell viability and sphere formation	Breast, colon	[228, 229]
GDC-0449	Hedgehog signaling	Patient trial: loss of CSCs + gemcitabine	Pancreas	[230, 231]
MK-0752	Notch signaling	Combination therapy + docetaxel PT in mice	Breast	[232, 233]
Resveratrol	Natural product miRNA	Xenograft CL	CML, breast, and pancreas	[234–236]

[a]CL indicates that experiments carried out in cell line xenografts and not on patient material

case, we need to know the nature of genes that are responsible for the niche-independent survival of CSCs, with the implicit restriction that such genes should not be required for the survival of other (normal) stem cells.

Equally, inhibition of the self-renewal function of stem cells (Fig. 1.3a, c) should result in a depletion of the stem cell pool. Over time it is expected that the absence of stem cells would result in an "exhaustion" of the tumor and terminal differentiation/senescence of the bulk tumor cells, in the absence of a replenishment from the primitive epithelial cell pool. Such approaches are likely to be more effective when the "exhaustion strategy" is enhanced by addition of a second cytotoxic agent to kill either the luminal or TA cells directly.

By application of a differentiating agent it is also possible to convert the therapeutically refractory stem cells (indicated by a red arrow in Fig. 1.3c) to TA cells and thus render the differentiated cells susceptible to conventional killing mechanisms (apoptosis and necrosis shown). Such agents would frequently show no direct cytotoxic effects—when used purely for differentiation, but have frequently been misemployed as simple cytotoxic agents at higher doses, with predictable off-target effects. The latter is likely to be a major drawback for this form of therapy, unless

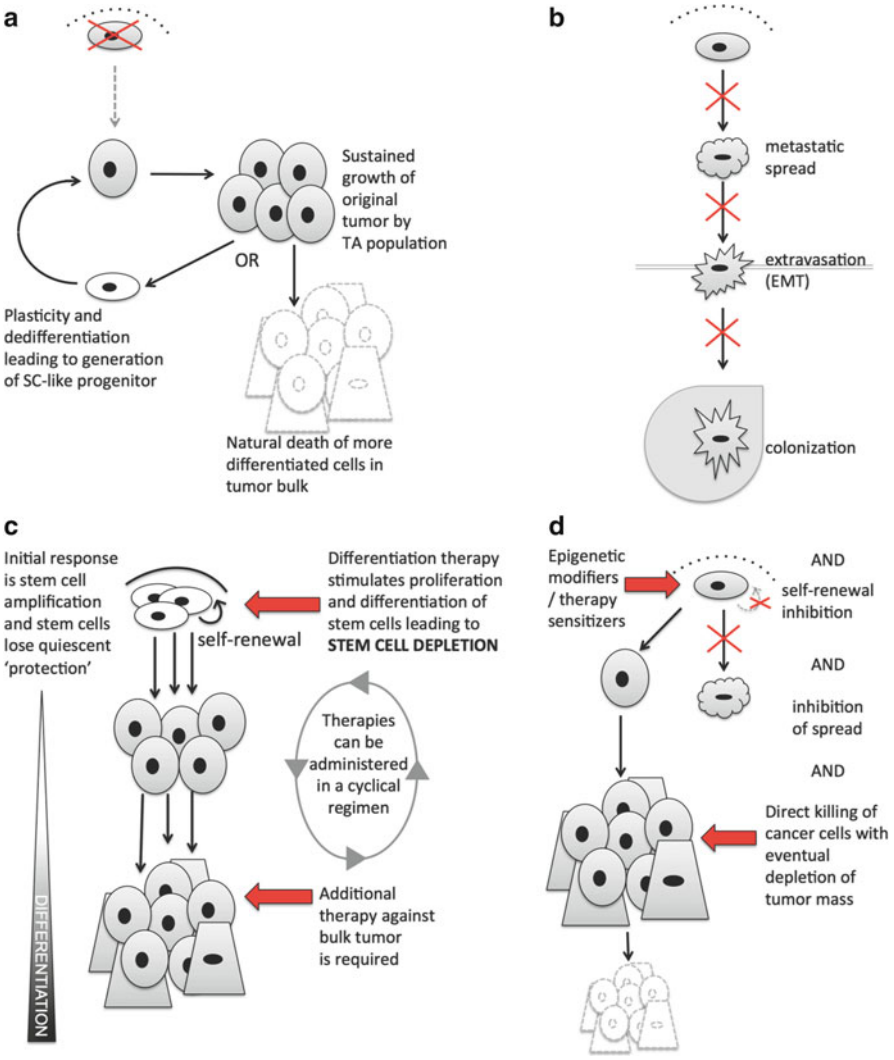

Fig. 1.3 *Strategies for cancer stem cell therapeutic development*. (**a**) *Destruction (direct killing) or differentiation (stem cell pool depletion) of the CSC pool*: Eventually, this treatment will result in a tumor, which may continue to proliferate, but will eventually become "exhausted." This treatment would show no short-term effects on tumor growth, and the time to exhaustion is unknown and may vary between tumors. (**b**) *Inhibition or blockade of tumor initiation and spread via CSCs:* Specific targeting of CSC properties such as EMT and colony formation (initiation of tumors at metastatic sites) could confine tumors to the prostate. However, escape from such inhibition by activation of alternative pathways could limit its effectiveness. (**c**) *Combination therapies after "activation" of CSC by destruction of tumor bulk:* This can either be achieved by cyclical application of conventional therapies as a monotherapy, or a combination of a more CSC-directed agent with a current therapy, which is cytoreductive. (**d**) *Combination therapies with epigenetic or fate-modifying agents:* Here, the initial treatment to break stem cell quiescence and/or resistance need not be toxic, and could result in a short-term *increase* in tumor markers. However, the resultant tumor would then contain higher numbers of susceptible cells for more conventional therapies (after Frame and Maitland [183])

Table 1.5 In vitro and in vivo tests for cancer stem cell therapeutics

Proposed effect	Experimental read out	Consequences
In vitro assays		
Induction of apoptosis	Annexin V/caspase expression, DNA fragmentation	Direct cell killing of CSCs
Induction of differentiation	Loss of expression of SC markers, expression of early and late luminal markers such as PAP and PSA	Depletion of SC pool: generation of cells susceptible to conventional agents
Inhibition of self-renewal/ asymmetric division	Loss of CSC markers, increase in committed basal cell markers	Loss of ability to form viable primary/secondary colonies or prostatospheres
Suppression of metastatic potential	Boyden chamber invasion/ motility assays	Organ confined tumor in chronic manageable form
In vivo assays		
Established tumor treatment with single agent	Tumor shrinkage/eradication	No or long delayed effect
Established tumor treatment with antiproliferative and CSC agent	Tumor shrinkage/eradication	Shrinkage plus delay in relapse/eradication
Ex vivo treatment and reimplantation	Tumor induction	No tumor induction, delay in growth, and unsustained growth

specific differentiation-controlling functions in prostate can be identified: the ability to differentiate, albeit aberrantly, seems to be hard wired into many tumor types.

A third option (Fig. 1.3b) could be the inhibition of the invasive function of the CSC, if they are indeed responsible for primary dissemination of tumors to metastatic sites. Such a strategy would require long-term application and could be susceptible to the development of new invasive phenotypes.

Finally, a commonly employed strategy has been to adopt an empirical approach, by testing the activity of existing agents against stem cells. The paradox here is that many of these agents would have been failures in traditional anticancer assays, which are based on the enhanced killing of cancer cells, relative to normal. If stem cells are in a largely quiescent state, then they will be impervious to such drugs. Some such agents are listed in Table 1.4.

It is conceivable that the repair and renewal properties of cancer stem cells within a tumor can be exploited to undermine their treatment resistance. If, as we suspect, the quiescent CSC population is activated by the destruction of the tumor mass, to produce new repopulating transit-amplifying cells, this may be their Achilles' heel. By inducing widespread cell death, there may be a window of opportunity to apply CSC therapies. This strategy of course assumes that all CSCs react to "repopulate" the tumor, which is perhaps too simplistic an hypothesis. Remaining CSCs could then regenerate the tumor in the post-treatment environment. It is perhaps only after such combination therapies are tested that the demands on cellular dynamics and its impact on treatment will be revealed.

There may be an example of just such a reaction in the outcome of the REDUCE and PCPT clinical trials [192]. In December, 2010, the FDAs' Oncologic Drugs Advisory Committee (ODAC) voted against recommending dutasteride (Avodart, GlaxoSmithKline) for an indication of reducing prostate cancer risk, because in the view of the ODAC members "the risk for more aggressive tumors outweighed the potential for chemoprevention." ODAC then recommended against prostate cancer chemoprevention labeling for the 5-alpha reductase inhibitors—dutasteride and finasteride. With a knowledge of the existence of an androgen-insensitive cancer stem cell in the pre-existing tumors in the men who did develop fatal metastatic cancers in these trials, then the phenotype of the observed cancers, which arose in an androgen response modified environment (undifferentiated/aggressive), is less surprising.

Clinical Applications of Prostate Cancer Stem Cell Therapies

As discussed above, the ideal CSC therapeutic should affect both the SC and the growing component. The therapeutic index would be both tumor shrinkage and lack of recurrence (and increased survival). This remains both unlikely and probably unattainable. In fact, a targeted CSC therapy would have neither a medium nor long-term effect on tumor size, but might prevent spread and degree of secondary disease, which is of course the fatal lesion in advanced prostate cancer. The use of combination therapies (Fig. 1.3c, d) to shrink tumor bulk *and* to remove the CSC component is therefore preferable, but the most effective means of developing such combinations are still in their infancy. For example, should a cytotoxic therapy be used *before* the putative stem cell treatment, in a manner similar to adjuvant androgen withdrawal before radiotherapy [193] or *subsequently* to stem cell eradication? The enrichment for a CSC population after cytotoxic treatment and a possible release of quiescence or destruction of a protective niche as part of a CSC "repair response" in the cancer suggest that a primary cytotoxic treatment could make CSCs more susceptible to treatment. Equally, the collateral damage after strong cytotoxic therapy to the immune response and tissue integrity argues that the CSC therapy should perhaps be the primary treatment.

Next, which type of patients *will* we be allowed to treat? Most phase 1/2 clinical trials are carried out on terminal CRPC patients, but after multiple rounds of chemotherapies is this form of prostate cancer a single disease or does it consist of many variants, compared to a primary untreated cancer [194, 195]? If advanced prostate cancer is truly multifocal, a highly targeted therapy could only eradicate a single clone of CSCs. The data from Luo et al. [195] and from high-throughput genome sequencing [196] suggest that this is rare, and that most disseminated disease probably originates by selecting variants of a founder tumor. Therefore, if the targeting were able to eliminate a "founder mutation," assuming that it was still essential for the survival of the variant CSCs [195], then the chances of success are higher.

One can also question the need to kill the CSCs: introducing the concept of quiescence-breaking or differentiating agents as the co-treatment to make the CSC population more susceptible to standard chemotherapies [197]. This has been immensely effective in some tumors such as acute promyelocytic leukemia [198, 199].

Lastly, what is the correct agent to achieve the targeting of CSCs, to overcome the potential resistance via enhanced small molecule effluxing, and/or the location of the CSCs within the tumor mass? For example, are the CSCs at the invasion front, as in some mouse models, or deep within the tumor mass at sites inaccessible to more bulky therapies such as monoclonal antibodies, nanoparticles, and viruses—or perhaps both? To resolve all of these contentions requires considerable experimental effort, before translation to patients. Perhaps the greatest danger to this field is the *premature* application in humans, ahead of a deeper knowledge of the consequences of the therapy. Gene therapy for inherited immunodeficiency has already been affected by this triumph of enthusiasm over ability [200].

Conclusions

The existence of cancer stem cells, whilst suspected for many years, is a relatively recent development in our understanding of human tumors. Despite increasing evidence pointing to the critical role of these relatively rare cells, controversy about their phenotype and responses to prostate cancer treatment remains. In some ways, the behavior and phenotypic nature of these cells may be seen to contradict the most established dogmas in our understanding of prostate cancers. If the tumor-initiating cell in human prostate is indeed a nonluminal/basal type, then it will be impervious to the long-established hormonal-based [173] and current treatments, which only rarely effect a cure in High Gleason grade tumors. Strategies to completely block the hormonal response are unlikely to bring substantial benefit on this basis. Our current treatments are influenced by a reliance on the few established cell lines and long-term xenografts employed in therapeutic development. One of the major lessons from stem cell studies has been to reaffirm the cellular and genetic heterogeneity of prostate tumors—even in these same cell lines. Ultimately, our strongest experimental paradigm is the patient. The treatment strategies of the next decade should inevitably take this into account, if we are to improve the disappointing medical responses we see at present.

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Chapter 2
Role of Reactive Stroma in Prostate Cancer

Rebeca San Martin and David R. Rowley

Abstract Reactive stroma initiates at sites of epithelial damage to mediate tissue repair and restore homeostasis. Genomic instability of epithelial cells at sites of early lesions such as prostatic intraepithelial neoplasia produces a similar breach of the epithelial barrier and an initiation of reactive stroma. Reactive stromal cells, termed myofibroblasts and carcinoma-associated fibroblasts, have been shown to originate potentially from several sources including tissue fibroblasts, resident stromal stem cells, vascular cells, and marrow-derived mesenchymal stem cells. Several growth factors such as transforming growth factor-β and interleukin-8 induce reactive stroma and regulate several downstream factors expressed in reactive stroma. Reactive stroma in prostate cancer is heterogeneous, and the amount of reactive stroma is predictive of disease progression. The heterogeneity of cells in reactive stroma is possibly a key aspect of the tumor-promoting properties. It is likely that reactive stroma biology is an important aspect of tumor progression to metastasis and acquired therapeutic resistance. Targeting the tumor microenvironment reactive stroma together with direct targeting of cancer cells may represent an effective therapeutic approach for the treatment of prostate cancer.

Homeostasis Control and Reactive Tissue

Homeostasis is a critically important concept in biology. The term homeostasis can be broadly defined as the continual maintenance of balance in environmental conditions of the cell and tissue throughout life. All tissues and organ systems, in some manner, are involved in this homeostasis control, which necessarily involves

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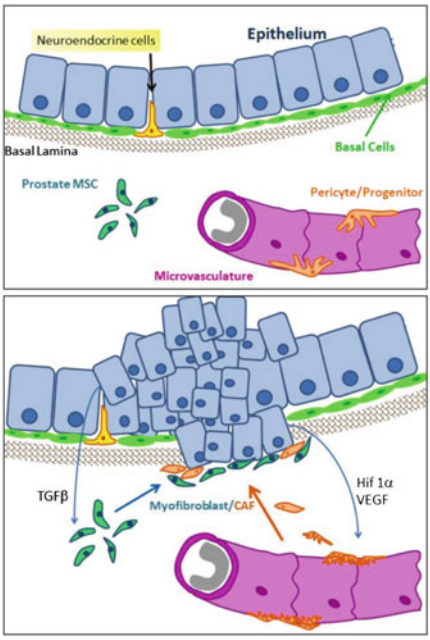
coordinate interactions between many cell types. The balance achieved by these interactions is critical for the maintenance of adult differentiated tissue function.

The key biological principle that orchestrates these emerging mechanisms in cells and tissues is the inherent biological concept of survival of self and survival of the species. Owing to this fundamental principle, once homeostasis has been compromised in a tissue, the biology of priority is rapidly adjusted from one of adult differentiated gene expression and function to one of repair and restoration of homeostasis, in which tissue and cell interactions are orchestrated to promote survival. Why is this principle so important to the understanding of the role of reactive stroma in prostate cancer? This fundamental principle is the basis for understanding and studying the role of the tumor microenvironment during tumor progression and metastasis. It follows that a change in the biology of priority to one of survival would necessitate changes in cell phenotypes, in gene expression profiles, and in tissue structure in order to service the new priority of tissue repair. As will become apparent in this chapter, the stromal compartment of cells lying adjacent to epithelium is uniquely designed to foster a rapid repair of the epithelium. Accordingly, the role of the reactive stroma and, indeed, the whole of the tumor microenvironment is not to service the needs and progression of the carcinoma. Rather, the responses of the stromal compartment have evolved to service the need to restore homeostasis back to that of the normal functioning tissue. In this regard, the important declaration by Dvorak that tumors are "wounds that do not heal" is an accurate perspective that helps greatly in investigating the role of the tumor microenvironment that coevolves with the carcinoma [1].

The prostate gland is a typical epithelial glandular tissue, in that it is constructed with glandular acini composed of polarized epithelial cells that secrete into the acini lumen. A continuous layer of secretory epithelial cells resides with basal epithelial cells and the intermittent neuroendocrine epithelial cells, all sitting on a basal lamina of extracellular matrix (Fig. 2.1). Important for this discussion, it should be noted that all epithelium, irrespective of location or function, exhibit a key barrier function in the tissue or organ [2]. The epithelial layer is continuous and the surface of the epithelium is in contact with the outside environment. This surface has the potential to harbor microorganisms and foreign proteins. Hence, the surface of epithelium exhibits a protective biology and is either covered with keratin squames, as in stratified squamous epithelium of the skin, or covered with mucous secretions as in the pulmonary and gastrointestinal tract epithelium. Whether it be keratin squames, mucous, or other secretions, these products of epithelium serve a protective innate immune function as a barrier to outside invaders [2]. The continuous epithelium also exhibits apical junctional complexes that serve both adherence as well as barrier functions. In addition, all epithelium reside on a basal lamina of matrix that is also continuous. Accordingly, while all epithelium are designed to secrete and/or absorb materials specific for the function of the tissue where it resides, the epithelial layer also provides a key barrier function that is critical to the evolved need for survival of self and survival of the species. It follows that if trauma or disease results in a breach of this epithelial barrier, this event would elicit a rapid and specific response to the underlying stromal compartment that is designed to



In normal prostate, the epithelium forms an important barrier between the external environment (lumen of the acini) and interior stroma via apical junctional complexes and a continuous intact basal lamina. Prostate resident mesenchymal stem cells (MSCs) and the microvasculature remain quiescent in steady state conditions.

In premalignant prostate cancer, proliferative changes in epithelium and loss of polarity accompanies a breach in the basal lamina. Cytokines and other factors from the epithelium trigger activation of a reactive stroma myofibroblast/CAF-like phenotype, derived potentially from resident mesenchymal stem cells, adjacent microvasculature, and the bone marrow. These responses are similar in damaged normal epithelium and the reciprocal wound healing response where a breach in epithelial barrier has occurred.

Fig. 2.1 Evolution of reactive stroma at sites of epithelial damage or pre-neoplastic changes

return the tissue or organ back to normal homeostasis [3]. Formation of a carcinoma in situ or a premalignant condition such as prostatic intraepithelial neoplasia (PIN) results in a loss of normal epithelial structure, in which the architecture of the epithelial layer is compromised (Fig. 2.1). During PIN, the epithelium loses its characteristic polarity, epithelial piling occurs, and apical junctional complexes that are so critical to the barrier function are altered [4–6]. Moreover, the basal lamina exhibits changes suggestive of degradation. Hence, coordinate with genomic instability and evolution of the premalignant conditions in the epithelial cell layer, a fundamental breach in the barrier function of epithelium occurs. It follows that a reactive response in the adjacent stroma would be predictable.

During normal wound repair in epithelial tissues, the breach of the epithelial barrier leads to production of cytokines and chemokines from the damaged epithelium [2]. Many factors function interactively to recruit neutrophils, macrophages, and other effector cells. Moreover, stromal fibroblasts and myofibroblasts function to remodel the extracellular matrix and eventually contract to aid in wound closure [3]. During this repair state, remodeling of the stromal compartment also takes place leading to activation and repair of damaged blood vessels, sprouting of new vessels from existing vessels (angiogenesis), and remodeling of nerve networks. Therefore, the importance of the coordinated response put into place within the stromal compartment is obvious: to ensure the rapid repair of a damaged epithelial barrier and, therefore, to promote the likelihood of survival.

The Coevolution of Reactive Stroma with Prostate Cancer

PIN results in a disrupted epithelial barrier in the prostate gland. As such, focal regions of a prototypical reactive stroma response are observed in approximately 50 % of PIN foci [7]. This initial stromal response is typified by expansion of vimentin-positive fibroblasts that reside immediately next to the basal lamina of the prostate epithelium. These cells also begin to express pro-collagen type I, common to wound repair stroma. Such cells have been termed carcinoma-associated fibroblasts (CAFs) and play a critical role in regulating carcinoma progression (Fig. 2.1). During progression of PIN to well-differentiated adenocarcinoma, the reactive stroma evolves to ~50 % CAFs and 50 % myofibroblasts at Gleason 3 foci [7]. This converts to mostly myofibroblasts at Gleason 4 foci. The myofibroblasts are vimentin and smooth muscle α-actin (α-SMA) positive but express lower levels of α-SMA compared to the smooth muscle cells in the normal human prostate gland [7, 8]. These myofibroblasts are also positive for fibroblast activation protein (FAP), tenascin-C, and pro-collagen I, suggesting that they are active in matrix turnover and remodeling during cancer progression. Select foci of PIN also overexpress transforming growth factor beta (TGF-β1), although the direct spatial localization of TGF-β1-positive PIN foci has not been correlated with foci of reactive stroma [7]. Coordinate with the evolution of reactive stroma, there is a remodeling of the nerve network and active neurogenesis and axonogenesis in the tumor microenvironment of human prostate cancer [9].

The relationship between CAFs and myofibroblasts is not yet understood. It is not known whether these cells represent different downstream phenotypes of the same stem/progenitor cell type of origin or whether they are separate lineages of cells. Recent studies have shown, however, that the heterogeneity of the reactive stromal cells with respect to their responsiveness to TGF-β is associated with an enhanced promotion of experimental prostate cancer [10, 11]. The types and sources of reactive stroma cells and their interactions are complicated and are not yet well understood. Moreover, how these stromal cells interact with immune system components, vessels, matrix, and nerves in the tumor microenvironment is not known and is likely to involve a complex network of signals and co-regulation [12–15]. Yet, these interactions and the biology that evolves are likely to be important for the development of novel prognostic and therapeutic approaches [16]. It is becoming clear that once CAFs acquire different properties as compared to normal fibroblasts, they seem to maintain these properties even when separated from cancer cells [17]. The biology of CAFs has permitted key studies that show the potent cancer-inductive potential of CAFs in prostate cancer models [16, 18, 19].

The Association of Reactive Stroma with Prostate Cancer Progression

Both CAFs and myofibroblasts are tumor promoting, as might be expected given their similarities to fibroblasts and myofibroblasts in the wound repair environment [10, 11, 19–23]. Analysis of human prostate cancer tissue for markers of reactive

stroma has shown that the relative volume of reactive stroma is a significant predictor of disease progression to biochemical (prostate-specific antigen levels) recurrence after prostatectomy [8, 24]. In addition to volume of reactive stroma, markers such as vimentin are useful in predicting what patients might progress more rapidly. Patients who had higher vimentin levels, yet identical Gleason scores, progressed to recurrent disease more rapidly [25]. Similarly, elevated expression of matrix metalloproteinase-9 (MMP-9) in stromal cells is associated with biochemical recurrence in prostate cancer [26]. Moreover, the volume of reactive stroma is also a significant predictor of prostate cancer-specific death [27].

The differential biology of how reactive stroma affects cancer progression is congruent with observed changes in gene expression. Gene expression profiling of reactive stroma as compared to normal stroma has also shown significant changes in gene expression patterns. When laser-dissected regions of reactive stroma grade 3 (50 % or more of tumor is reactive stroma) were compared to normal prostate gland stroma regions, over a 1,000 unique genes showed significant differences in expression levels (544 overexpressed and 606 underexpressed) [28]. Gene ontology analysis in this study suggested that neurogenesis and DNA damage and repair pathways were most prominently affected. Other pathways included metabolic changes and stem cell activity. Another study showed that 671 transcripts were elevated and 356 were decreased in prostate cancer-reactive stroma [29]. Gene ontology analysis suggested genes involved in prostate morphogenesis were enriched and cell cycle genes were decreased. An additional gene expression profiling study pointed to the differential expression of osteogenic proteins in reactive stroma in prostate cancer [30]. Changes in gene expression patterns are observed in the reactive stroma tumor microenvironment in other cancers as well. In ductal carcinoma in situ (DCIS) of the mammary gland, changes in gene expression patterns in both the stromal cells and macrophages have been reported [31]. Changes in invasive breast cancer showed altered patterns in reactive stroma suggestive of matrix remodeling and proteolytic changes [32]. Moreover, changes in gene expression patterns in the reactive stroma in breast cancer have even been shown to be predictive of response to presurgical chemotherapy [33]. An analysis of select genes in the reactive stroma of hepatocellular cancer showed that amount of reactive stroma was predictive of recurrence and suggested regulation of angiogenesis, matrix remodeling, and immune activity [34]. Overall, it is becoming evident that changes in gene expression profiles within the tumor microenvironment show defined patterns of genes that are more predictive of metastasis behaviors [35].

In addition to transcriptional changes, there appears to be differential regulation by micro-RNAs and epigenetics in the reactive stroma phenotype as well. Both miR-15 and miR-16 were downregulated in prostate cancer-reactive stroma, while re-expression of these attenuated the cancer-promoting activity of reactive stroma [36]. It is likely that epigenetic changes in methylation occur within reactive stroma in prostate cancer [37]; however, this has not been thoroughly studied.

In light of the developing literature that suggests reactive stroma is associated with major shifts in oxidative stress and inflammation, the altered gene

expression patterns reported so far point to fundamental processes of repair and renewal of stromal tissue. This is consistent with the hypothesis that reactive stroma coevolves with cancer as a compensatory event and, importantly, that this coevolution is likely to affect resistance patterns to therapeutics. Although reactive stroma within a particular cancer type shows changes as compared to normal, the gene sets seem to be somewhat distinct for the tissue/tumor type. For example, a comparison between gene sets in breast and prostate cancer-reactive stroma revealed distinct expression patterns, suggesting that different tumor types exhibit distinct changes in gene expression within the reactive stroma [38]. Some of the differences in gene expression patterns are likely the result of the heterogeneity of different reactive stroma cell types within a particular tumor. In any event, the data suggest that reactive stroma, in general, is promoting cancer progression and the volume and specific markers are significantly correlated with cancer progression. Less understood are the cell types of origin for reactive stroma and how this might relate to the heterogeneity of reactive stroma cell types and their biology during cancer progression.

The Origin of Reactive Stroma

Critical questions have emerged over the last 10 years as to the cell type or cell types of origin for the generation of reactive stroma in wound repair, chronic fibroses, and cancer. Moreover, the general nature and normal function of these reactive cells in tissue homeostasis are not fully understood. It is not likely that a specific type of reactive fibroblast evolved to just provide a reactive stroma microenvironment to service carcinoma cells. The term "carcinoma-associated fibroblast" was generated because these cells were adjacent to cancer cells, could be derived from tumor tissue, appeared to have differential expression of reactive stroma markers, and, importantly, exhibited differential tumor-inductive ability when tested either in vitro or in vivo [19, 39, 40]. It is unlikely that this cell type evolved solely to serve a role in cancer; rather, it is likely that these cells emerge at sites of disrupted epithelial homeostasis and breach of the epithelial barrier. This becomes an important issue to understand as we learn more about the stem and/or progenitor cells that evolve to reactive stroma CAFs (reactive fibroblasts) and/or myofibroblasts. Such knowledge could aid in developing therapeutic approaches to target the differentiation of these cells. Many studies now point to the likelihood of multiple sites and cell types of origin that produce these reactive stromal cells.

A recent study using mouse models has shown that CAF-like cells, which are FAP-positive "tumor-associated fibroblasts (TAFs)," are likely recruited from marrow-derived mesenchymal stem cells, while "fibrovascular stroma" such as myofibroblasts and endothelial cells are derived from adjacent adipose tissue in breast and ovarian cancer models [41]. Studies that have directly compared

tumor-derived TAFs to mesenchymal stem cells (MSCs) show that they share several characteristics and common sets of differentially expressed genes and suggest that TAFs should be viewed as a subset of MSCs with special features [42]. Several studies point to the recruitment of tissue-derived MSCs as a key source for CAFs and myofibroblasts. A key study examining acute tissue injury concluded that "profibrotic cells," which transiently express a disintegrin and metalloprotease 12 (ADAM-12), originate in the perivascular wall and give rise to "collagen overproducing cells"—myofibroblasts [43]. Cancer recruits MSCs from local tissue and from circulating pools [44, 45]. MSCs migrate toward carcinoma cells, and stromal cell derived factor-1 (SDF-1, CXCL-12) expression in MSCs is required for this migration [46]. Similarly, a myocardial infarction model showed that SDF-1 was critical for MSC migration to the wound and expression of cytokines [47]. Moreover, human marrow-derived MSCs are induced to CAFs by exposure to "tumor conditioned media," and these CAFs also express SDF-1, exhibit myofibroblastic features (α-SMA positive), and exhibit tumor-promoting activity [48]. In a gastric cancer model, ~20 % of CAFs in the tumor were derived from bone marrow and again SDF-1 was implicated in their recruitment and biology [49]. Lung cancer cells induce MSCs derived from adipose tissue to express periostin, which mediates both the subsequent differentiation of the MSCs to myofibroblasts as well as their tumor-promoting biology in vivo [50]. Similarly, lung-derived MSCs were shown to be "unique" from lung fibroblasts and functioned to regulate much of wound repair and effector T-cell immune responses [51]. MSC biology also affects immune responses. MSCs are mobilized from the bone marrow and can be recruited to tumors where they can also have immunosuppressive functions that are tumor promoting; however, they can sometimes exhibit tumor-inhibiting properties as well [52, 53]. Moreover, MSCs affect macrophage function [54], dendritic cell function [55, 56], and the amount of regulatory T cells in the tumor microenvironment in a breast cancer model [57]. Indeed, MSCs may affect many different aspects of immune responses [58]. MSCs may also play a role in response to therapy and therapeutic resistance. Radiation in a mammary tumor model resulted in increased recruitment of MSCs [59]. Accordingly, MSCs or MSC-like cells most likely are recruited both from local sources, including the vascular wall, as well as from bone marrow. Less clear are the different types of cells in the tumor microenvironment into which MSCs are capable of differentiating and the specific function these cells serve. Finally, how MSCs interact with other cell types within the tumor microenvironment and the role they may play in response to therapy are not understood. Within the theme of homeostasis control, it is likely that MSCs and their derivatives in reactive stroma are capable of exhibiting both tumor-promoting and tumor-inhibiting functions and that the balance of these functions is dependent on other cell types, the milieu of growth regulatory factors, the state of immune response, and the specific tumor type.

In addition to MSCs, other cell types have been implicated as potential progenitors for CAFs and myofibroblasts. In liver diseases including cancer, myofibroblasts

arise from perivascular stellate cells [60]. Similarly, pro-fibrotic stromal cells that are activated in acute injury originate from cells in the perivascular space [43]. The origin of reactive fibroblasts (CAFs) from the vascular compartment is intriguing for many reasons. The postcapillary venule side of the microvascular network is the vascular segment most involved with inflammatory reactions during disrupted tissue homeostasis [61]. This segment is where control of tissue fluid occurs during inflammation and is most sensitive to regulators such as histamine, serotonins, and bradykinins. An attractive hypothesis is that this segment may house or produce stem/progenitor cells, which boil off of the postcapillary venules in reactive situations, in order to form an initial reactive stroma for supporting the initial phases of tissue repair. These phases include formation of granulation tissue and new vessels through angiogenesis, representing classic wound repair. Each of these features has been noted at sites of initial reactive stroma formation in PIN at the base of epithelial acini, where there also exists a strategic placement and abundance of microvasculature [7]. Moreover, TGF-β, a potent regulator of reactive stroma, induces transition of endothelial cells to a mesenchymal mural cell (smooth muscle actin positive) in a Snail-dependent manner [62]. It is possible that such activated mural cells may function to be the first wave of CAFs observed during initial reactive stroma formation.

CAFs and myofibroblast-reactive stromal cells may result from epithelial to mesenchymal transition (EMT). Matrix metalloproteinases (MMPs) may induce epithelial cells through EMT to become myofibroblasts at sites of fibroses and within the tumor microenvironment [63, 64]. In a mouse model of Crohn's disease, EMT of intestinal epithelial cells was implicated in the fibroses; however, cells exhibited both epithelial and mesenchymal markers [65]. Circulating fibrocytes, which share characteristics with monocytes and fibroblasts, form a mesenchymal-like reactive stroma at sites of wound repair and fibrosis, although the biology of these cells is not well understood [66].

Together, these studies suggest that CAFs and myofibroblasts can originate from several sources, including MSCs, the vascular wall, circulating cells of bone marrow origin, and possibly from resident epithelial cells (Fig. 2.1). It is likely that sites of reactive stroma in various diseases and wound repair involve cells that originate from these various sources and that these cells exhibit an interactive biology that evolves to provide the most optimal repair for the specific defect or disorder.

Regulatory Mechanisms and Reactive Stroma Biology

Additional questions have emerged regarding the identification of key mechanisms that induce and regulate the formation of reactive stroma. Again, such mechanisms could be targeted with new therapeutic approaches. Little is understood about the specific mechanisms of recruitment and activation of CAFs and myofibroblasts. Several factors including TGF-β have been implicated in the induction of myofibroblasts. TGF-β has activities in the microenvironment that are both tumor promoting

and tumor inhibiting. TGF-β promotes myofibroblast differentiation as well as reactive stroma biology and induction of tumor growth in some experimental systems, including prostate cancer [14, 22, 67, 68]. TGF-β-induced pathways lead to altered expression of many genes in prostate cancer-derived myofibroblasts, including smooth muscle α-actin, calponin, and tenascin-C [69]. TGF-β inhibits stromal cell promotion of cancer and loss of signaling functions to stimulate the tumor-inductive properties of reactive stroma and T cells [70–72]. The heterogeneity of response to TGF-β in stromal cells potently promotes prostate cancer tumorigenesis [10–12], which may explain some of the previously paradoxical results. It is clear that TGF-β is a very potent regulator of reactive stroma in wound repair, fibrosis, and other proliferative diseases. TGF-β induces expression and secretion of several pro-angiogenic and pro-tumorigenic factors including connective tissue growth factor (CTGF) and fibroblast growth factor beta (FGF-2) in prostate stromal cells [23, 67]. The role of the TGF-β/CTGF axis in regulating reactive stroma has also been implicated in hepatocellular cancer [73]. The significance of FGF-2 in prostate cancer microenvironment is further supported by studies of microRNAs. Both miR-15 and miR-16 are downregulated in human prostate cancer microenvironment [36]. Downregulation of these miRNAs in CAFs led to loss of repression of FGF-2 and FGFR1 and promoted tumor cell proliferation, migration, and survival.

The expression of CXCL-8 or interleukin-8 (IL-8) is also upregulated in human prostate cancer and correlates with disease progression [74]. We have shown that IL-8 and its murine homolog, keratinocyte chemokine (KC), are potent inducers of reactive stroma phenotype in benign prostatic hyperplasia, including the expression and spatial deposition of tenascin-C [75–77]. Although IL-8 has been studied in many proliferative and inflammatory disorders and appears to regulate many components of reactive stroma biology, its specific role in prostate cancer progression is not yet understood.

Other chemokines have also been implicated in the interactions between reactive stroma and cancer. In prostate cancer, the expression of CXCL-14 was elevated in CAFs of most cancers analyzed and promoted prostate cancer xenograft tumor growth, macrophage infiltration, and angiogenesis [78]. In mammary cancer models, interleukin-6 (IL-6) was secreted by reactive stroma cells and mast cells and induced EMT in cancer cells [79]. IL-6 is also secreted by carcinoma cells in prostate cancer and activates CAFs to secrete matrix metalloproteinases (MMPs) that play a role in EMT, tumor progression, and metastasis in an experimental model [80]. In addition to CXCL-14 and IL-8, several additional chemokines have been implicated in mediating "crosstalk" between carcinoma cells and CAFs, including CXCL-12 (SDF-1), CCL2, CCL5, and CCL7, which are also associated with tumor progression and malignancy [81]. Of these, CXCL-12 appears to be a key factor in reactive stroma biology [82]. CXCL-12 induces recruitment of marrow-derived vasculogenic progenitor cells, thereby promoting vasculogenesis [83]. CXCL-12 activity is mediated through the CXCR4 receptor, which is likely to be critical for cancer cell survival, growth, and metastasis [84]. Both CXCl-12 and CXCR4 can be regulated by cyclooxigenase 2 (COX-2) and associated prostaglandins, other key mediators of inflammation [85].

Several growth regulatory factors associated with wound repair and tissue remodeling have been implicated in the biology of reactive stroma in cancer. Hepatocyte growth factor (HGF) secreted by reactive stroma promotes invasion of esophageal cancer cells [86]. Paracrine signaling between cancer and stromal cells through stimulation of Hedgehog pathways in stromal cells has also been implicated, and this may involve stimulated IL-6 expression in stromal cells as well as stimulation of angiogenesis [87–89]. In prostate cancer, stimulation of paracrine Hedgehog signaling in reactive stroma led to myofibroblast promotion of tumor growth [90].

Several extracellular matrix-associated proteins have also been implicated as being important in the tumor microenvironment of reactive stroma. Tenascin-C, a matrix-associated glycoprotein, is a marker for reactive stroma in prostate cancer [7, 91] and is elevated in most reactive stroma associated with cancer and in fibrosis. Tenascin-C is a potent factor in the activation of innate immunity and inflammation [92, 93]. Moreover, tenascin-C is implicated in differentiation of myofibroblasts in reactive stroma and is an important mediator of fibrosis and of TGF-β action [94–96]. Tenascin-C has also been implicated in regulating branching morphogenesis of endothelial cells, new vessel development [97, 98], and in nerve repair processes [99]. In mammary cancer, tenascin-C expression by cancer cells is important for regulating metastasis initiating events in lung metastases [100]. Stromal cell expression of tenascin-C is also a critical component of metastatic capability, and this may involve prevention of apoptosis [101].

Other matrix-associated proteins that are likely involved in mediating the biology of the tumor microenvironment include secreted protein acidic and rich in cysteine (SPARC) and the ps20 protein (encoded by the WFDC1 gene) [102–106]. SPARC may involve formation of reactive stroma that is not tumor promoting and thereby may be involved in regulating the biological heterogeneity of reactive stroma [107]. Versican, another matrix protein, induces differentiation of stromal cells to myofibroblasts in the tumor microenvironment [108]. Versican together with hyaluronic acid (HA) promotes motility of prostate cancer cells [109]. HA is further implicated in the TGF-β induction of fibroblast to myofibroblast differentiation in reactive stroma [110]. Enzymes that regulate matrix biology are also important modulators of cancer progression. The matrix metalloproteinases (MMPs) are key regulators of matrix biology and cancer cell biology [111]. The expression of MMP-9 in prostate reactive stroma was correlated with biochemical recurrent disease [26]. The membrane-tethered MMP, termed MT-1-MMP, is responsible for collagen degradation of mesenchymal stem cells that is required for their invasion and differentiation in areas of tissue damage [112].

In general, most modulators of oxidative stress and inflammation have been implicated in the interactions between cancer cells and reactive stroma [113]. Cyclooxygenase-2 (COX-2) mediates the tumor-promoting activity of reactive fibroblasts in mammary cancer models, and this involves elevated MMP-9 activity [114]. COX-2 also modulates the CXCL-12/CXCR4 signaling axis in reactive stromal cells as discussed previously [85]. High COX-2 expression in stromal cells also promoted proliferation and VEGF production by cancer cells [115]. In a mammary

cancer study, high levels of reactive oxygen species induced fibroblast to myofibroblast conversion and this involved CXCl-12 activity [116]. Altered physiology regulated by elevated NOX-4 and generation of reactive oxygen species is downstream of the TGF-β-induced fibroblast to myofibroblast differentiation in prostate stromal cells [117]. Accordingly, there appears to be a close association between altered oxidative stress, the actions of growth factors that induce myofibroblast differentiation, and the formation of a tumor-promoting reactive stroma.

Importantly, the biology of MSCs, CAFs, and myofibroblasts seems also to be interactive with the biology of other marrow-derived cells such as mast cells and macrophages at sites of wound repair and reactive stroma in disease. It is likely that these reciprocal interactions affect a coordinate biology that integrates immune action with tissue repair and angiogenesis [118]. Macrophages cocultured with MSCs exhibit an alternative unique phenotype. This has led to the concept of a "mesenchymal stem cell educated macrophage" whereby interactions with MSCs induce an activated macrophage with a unique alternative phenotype [54]. Mast cells accumulation in the tumor microenvironment is associated with a more rapid tumorigenesis in a pancreatic cancer model [119]. Mast cells may also regulate immunosuppressive activity in the tumor microenvironment [120, 121], and their expression of IL-6 in the tumor microenvironment is also implicated in tumor-promoting biology [79] .

Androgens may play a role in the tumor-promoting biology of reactive stroma associated with prostate cancer. A subset of fibroblasts in the prostate gland expresses androgen receptor, and androgens regulated several growth factor pathways including TGF-β and Hedgehog [122]. Moreover, knockdown of androgen receptor in prostate stromal cells resulted in lower collagen deposition and a lower expression of several growth factors [123]. Although much remains to be understood, it is likely that androgen action in prostate gland stromal cells is involved in homeostasis of the stromal compartment and in the genesis of reactive stroma in prostate diseases.

Therapeutic Approaches that Target Reactive Stroma

Targeting the tumor microenvironment and the biology of reactive stromal cells is in progress and holds considerable promise, particularly when used in combination with direct cancer cell-directed therapeutics [124–127]. The tumor microenvironment exhibits a complex biology with many cell types that are likely to have a stable genome, unlike cancer cells. This concept may afford a more rational and sustainable therapeutic approach that could be less prone to therapeutic resistance. Altering the tumor microenvironment via "ligand targeted nanoparticle" in a murine mammary cancer model inhibited STAT-3 signaling that, when combined with a DNA vaccine to HER-2, resulted in an inhibited tumor growth and elevated immune surveillance [128]. Mice vaccinated against FAP (fibroblast activation protein) expressed in the reactive stroma exhibited inhibited tumor responses with

experimental melanoma, lymphoma, and carcinoma models [129]. Similarly, targeting FAP in murine lung cancer and colon cancer models resulted in decreased myofibroblasts, decreased vessel density, and inhibited tumor growth [130]. Another study that targeted FAP in a colon cancer model showed inhibition of primary tumor and inhibition of metastases [131]. In addition, the enzymatic activity of FAP, a peptidase, was used to target a protoxin activation at sites of reactive stroma in mammary and prostate cancer xenograft models, resulting in inhibited tumor growth in both models [132].

Considerable progress has been made in attempts to use the homing of MSCs to sites of injury or disease as a therapeutic approach. However, there are many variables and strategies in the preparation and evaluation method used to investigate this as a therapeutic approach [133]. The first study to use MSC cell homing to tumors focused on delivering an MSC-expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and resulted in apoptosis of cancer cells, reduced tumor growth, and reduced metastases [134, 135]. A similar study using a pancreatic cancer model showed the combined targeting of cancer cells with X-linked inhibitor of apoptosis protein (XIAP) and using TRAIL-expressing MSCs resulted in remission of tumors with greatly decreased metastases [136]. Together, these studies suggest that a combined therapeutic approach, which targets the cancer cells as well as the reactive stroma biology, and the use of stromal cells, which can be recruited to tumors as a method of delivery, may be useful approaches from which to build novel therapies.

How the targeting of reactive stroma could be used therapeutically for metastasis is not yet clear. Little is understood about the mechanisms by which reactive stroma in primary tumors affects the rate or patterns of metastasis. In addition, little is understood about the induction, cell types of origin, and biology of reactive stroma that forms in metastatic sites, as these too could be therapeutic targets. It is understood that bone is a rich source of growth factors and cytokines in metastatic cancer [137–139], and the biology of these factors could be targets. Prostate cancer cell lines that metastasize to bone induce MSCs to express factors, e.g., osteoprotegerin, that are pro-osteoblastic [140]. Whether these processes influence the evolution of metastases to bone or whether they could be used therapeutically are not yet understood.

Summary and Future Directions

We now understand more about the mechanisms that regulate reactive stroma induction and biology during cancer progression. In order to survive, cancer cells implement a complicated cascade of signaling mechanisms whereby reactive stroma from multiple cell types of origin is recruited. The interactions between these cells and immune components, vasculature and nerves, along with carcinoma cells ensue, which coevolve in heterogeneous patterns, with a net predictable biology that shares fundamental principles with wound repair. Reactive stroma cells appear to be

recruited from multiple sources including local stem/progenitors, local fibroblasts, and vascular-associated cells, and from circulating, marrow-derived MSC progenitors. The recruitment and activation of myofibroblasts and CAFs are multifactorial and complex. The heterogeneity of the reactive stromal cell types and their responses to key growth factors such as TGF-β is important, yet not well understood. How the biology of these reactive stromal cells affects tumor progression and metastasis is presently a very active area of research. It is likely that the multitude of mechanisms that regulate and mediate reactive stroma biology within the tumor microenvironment is even more complicated than the mechanisms that directly regulate cancer cell growth and metastasis. However, it is also likely that most, if not all, reactive stromal cells are genomically stable and, hence, their biology may be more predictable. It follows that owing to the stability and predictability, this biology may be more amenable to therapeutic targeting. Future directions should be directed to understanding the fundamental mechanisms of how reactive stroma regulates repair processes to return tissues to normal homeostasis. Since this biology is important for many other homeostasis processes, including wound repair, fibrosis, inflammation, immune surveillance, and angiogenesis, it is important to understand specific mechanisms. These mechanisms will likely be useful in new therapeutic approaches for disorders in addition to cancer.

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Chapter 3
The Role of Cholesterol in Prostate Cancer

Keith R. Solomon, Kristine Pelton, and Michael R. Freeman

Abstract Recent epidemiological and preclinical studies have shown that the steroidal lipid cholesterol is a clinically relevant therapeutic target in prostate cancer. This review summarizes the findings from human studies, as well as from animal models and cell biology approaches, which suggest that high circulating cholesterol can increase the risk of aggressive prostate cancer, while pharmacological cholesterol lowering may be protective against incident or advanced disease. A variety of molecular processes are described that have been implicated experimentally in this protective scenario or are otherwise plausibly connected. There is now sufficient experimental and observational evidence in humans to prospectively apply cholesterol-targeting strategies in selected patients to inhibit prostate cancer progression to the metastatic form of the disease.

Introduction

Prostate cancer (PC) is the second most common cause of cancer-related death in North American men [1]. Most cases of PC diagnosed in developed countries are successfully treated with surgery or other modalities; however, there are no successful treatments for advanced PC, particularly when the "hormone-insensitive" castration-resistant phenotype (CRPC) emerges following androgen deprivation therapy (ADT). One possible means to improve clinical outcomes would be to identify preventive strategies that reduce PC incidence or delay progression to CRPC.

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PC risk is affected by germline genomic variations that are not fully characterized [2–6] and revealed in the different disease rates that correlate with family history and ethnic origin [7–9]. Environmental factors play a major role. The most robust evidence of environmental effects is seen with rapid increases in PC incidence in migrants from poor to more developed countries [10–13]. Diet is thought to be a primary basis for geographical variations in PC risk [14–18]. The relevant dietary components are debated, but include hormones, estrogenic compounds, animal products, carcinogens, excess caloric intake, as well as high levels of fat and cholesterol.

Cholesterol is a steroidal lipid that is an essential component of animal cells and makes up about one-third of the lipid content of the plasma membrane [19, 20]. Its unique steroid chemistry exerts dynamic control over membrane structure and fluidity [19]. Due to its relative ease of oxidation, cholesterol is cytotoxic at high concentrations and is an essential precursor in the biosynthesis of steroidal hormones. Thus, cellular cholesterol content is carefully regulated, even in the face of wide fluctuations in serum cholesterol, by a variety of homeostatic mechanisms, including regulated uptake, synthesis, conversion to esters, bile acids and steroid hormones, as well as efflux from the cell [21–25]. Because of the complexities of this regulatory network, all cells are potentially vulnerable to disruptions in homeostatic control over cholesterol metabolism [26, 27].

A comprehensive consideration of the recent and historical literature supports the hypothesis that circulating and membrane cholesterol plays an important role in disease progression in PC. In the following pages we present these new data in the context of the older literature and discuss several molecular mechanisms that may account for observations in humans.

Epidemiologic Studies

A number of human studies provide support for the assertion that cholesterol plays a role in PC formation or progression: large population studies of the relationship between cholesterol and disease, observational studies of cholesterol and PC incidence, observational studies of cholesterol-lowering drugs and PC incidence and severity, and randomized trials of cholesterol-lowering drugs that report on cancer rates.

Most large population studies considering overall disease incidence, mortality, and cholesterol level published in the latter twentieth century do not consider PC in the analysis. Our calculation of the total number of PC cases in the combined literature from 1980 to 2000 is only 1,652 [28–35]. We have reviewed these reports previously and concluded that, when considered as a whole, they suggest a possible, modest association between low cholesterol and increased PC risk. Such an association is likely to result from the effects of pre-existing cancer, which can lower circulating cholesterol levels [36] (more on this point below). A more recent report, Kitahara et al. [37], describes a large prospective study in Korea that collected data

on 756,604 men and included 2,490 PC cases. This study found that the 329 men in the highest quintile of total cholesterol (≥240 mg/dL) had increased PC risk [HR 1.24 95 % CI (1.07–1.44), *p*-trend = 0.001] in comparison to the 366 men in the lowest quintile (<160 mg/dL).

Most of the studies cited above include limited numbers of PC patients, were generally of short duration, and typically did not report on late stage disease (except with respect to death). In contrast, a number of studies that specifically address the potential association between serum cholesterol and PC have large patient cohorts, many cases of advanced disease, and stratify disease by grade, thus permitting a more thorough analysis. The Asia Pacific Cohort Studies Collaboration 2007 [38] found a statistically insignificant larger number of deaths in the population with highest cholesterol, while Thompson and colleagues [39] observed no cholesterol–PC association ($n = 100$). In contrast, using case-control analysis of men in the Health Professionals Follow-up Study, Platz et al. [40] demonstrated that patients ($n = 698$) with low cholesterol had a reduced risk of high-grade PC [OR 0.61 95 % CI (0.39–0.98)]. In an independent study, the same group [41] examined 1,251 incident PC and found that men with cholesterol <200 mg/dL had reduced risk of high-grade disease. Mondul et al. [42] examined 438 incident PC and reported that men with cholesterol >240 mg/dL were at higher risk of developing high-grade disease than men with cholesterol <240 mg/dL. Hemelrijck et al. [43] analyzed 200,660 men of which 5,112 developed PC and found no cholesterol–PC association. However, in a subsequent report, this group [44] found that after eliminating the initial 3 years of follow-up, high density lipoprotein (HDL) was inversely associated with PC risk [HR 0.79 95 % CI (0.68–0.92), *p*-trend = 0.003], when comparing the highest (>63.8 mg/dL) to the lowest (<43.7 mg/dL) cohort of patient HDL levels using quartile analysis. Additionally, they observed that increased total cholesterol (TC)/HDL lipid ratios of >5.45 were associated with increased PC risk [HR 1.26 95 % CI (1.07–1.49), *p*-trend = 0.005] when compared to ratios of <3.44. Moreover, LDL (low density lipoprotein)/HDL ratios of >3.70 were found to be associated with increased risk in comparison to ratios <2.11 [HR 1.21 95 % CI (1.03–1.41), *p*-trend = 0.026]. Batty et al. [45], in a study that included 578 PC deaths, report a greater number of cancer deaths in the highest cholesterol tertile. Farwell et al. [46] demonstrated a significant relationship between total serum cholesterol and PC risk with a 204 % increased risk of high-grade PC [HR 3.04 95 % CI (1.65–5.60)] and 45 % increased overall risk of total PC [HR 1.45 95 % CI (1.07–1.97)] for patients in the highest quartile of total cholesterol (>237 mg/dL) in comparison to the lowest (<176 mg/dL). Shafique and colleagues [47] in a study including 650 men who developed PC found that cholesterol level was positively associated with the incidence of cancer with a Gleason score ≥8. In adjusted analysis, the association was largest [HR 2.28 95 % CI (1.27–4.10)] when the second highest cholesterol quintile (235.9–258.7 mg/dL) was compared to the first (<195.3 mg/dL). Using the Alpha-Tocopherol Beta-Carotene Cancer Prevention study cohort, Mondul et al. [48] examined a population of smokers including 2,041 who developed PC. These authors observed, after excluding the first 10 years of follow-up, that men with higher total cholesterol (≥240 vs. <200 mg/dL) were at increased risk

of advanced cancer [HR 1.85 95 % CI (1.13–3.03), *p*-trend = 0.05] and overall PC [HR 1.22 95 % CI (1.03–1.44), *p*-trend = 0.01]. Furthermore, in a comparison of men in the lowest to men with the highest quintile of total cholesterol/HDL ratios, there was a greater risk of advanced [HR 1.44 95 % CI (1.02–2.05)] and overall PC [HR, 1.20 95 % CI (1.02–1.41)]. In a prospective population-based study, Kok et al. [49] examined 2,118 men who reported having never used cholesterol-lowering drugs. Of the 43 PC cases, the adjusted analysis showed that higher LDL and total cholesterol levels were significantly associated with an increased risk of overall as well as advanced PC.

Although this literature is complex and somewhat contradictory, taken together it suggests that men with hypercholesterolemia are at increased risk for PC or aggressive disease.

Other epidemiologic data suggesting an association between cholesterol and PC risk comes from studies of cholesterol-lowering drugs (primarily 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors), collectively known as "statins" [50–62]. Statin drugs are used in medical practice to lower LDL levels and improve cardiovascular health. Statins inhibit the rate-limiting step in cholesterol biosynthesis in the liver and consequently reduce circulating cholesterol levels. Because statins interfere with an early step in cholesterol synthesis, they also reduce the production of upstream synthetic intermediates. Much of the activity of these agents seen in vitro can be attributed to a reduction in these non-cholesterol compounds. Statins act principally on the liver in humans, and most of their pleiotropic effects can be ascribed to the consequences of cholesterol lowering [36, 63]. We have argued from the known pharmacology and pharmacokinetics of statins that they are unlikely to accumulate in the prostate in sufficient concentrations for long enough periods of time to exert sustained local effects [36, 63]. The most plausible cause of effects on the prostate is in the potent cholesterol-lowering activity of statins; consequently, studies examining statins and PC risk are actually addressing the effect of *cholesterol lowering* on disease incidence or severity.

Platz et al. [59] analyzed potential statin drug effects specifically on PC in a large cohort that included 2,579 PCa cases and 316 cases of advanced disease. The adjusted relative risk of CRPC among statin users was 0.51 95 % CI (0.30–0.86) and of metastatic or fatal disease was 0.39 95 % CI (0.19–0.77) for statin users vs. non-users. These investigators also showed that the risk of advanced disease was lower with prolonged statin use. In contrast to the analysis of advanced disease, Platz et al. reported no association between statin usage and overall PC risk. Additional studies from several independent groups [54, 57–59] in large part supported the conclusion that statins reduce aggressive PC risk.

Despite a few inconsistencies in the literature [64–66], with two investigations reporting no association between PC risk and statin use [64, 65], one study showing a positive association [66] and another reporting that statin users had lower 5-year biochemical recurrence-free survival [67], a number of reports in the last several years reinforce the conclusion that statins can protect against PC.

Tan et al. [68] performed a study of 4,204 men who underwent prostate biopsy and showed that men who took statins (24.3 % of the total) were not as likely to test

positive on a digital rectal examination (5.3 vs. 8.9 %, OR 0.7, $p<0.01$) and less likely to have high volume PC (27.2 vs. 31.4 %, $p<0.01$) or a Gleason score ≥7 (61.4 vs. 72.4 %, OR 0.78, $p=0.02$). Adjusted analysis demonstrated that statin use at any time decreased incidence [RR 0.92 95 % CI (0.85–0.98)] and high-grade PC [RR 0.76, 95 % CI (0.67–0.85)]. Patients with >5 years of statin use also had a decreased incidence of high-grade PC [RR 0.75 95 % CI (0.53–0.94)] in comparison to those who never used a statin.

A retrospective study by Mondul et al. [69] of 2,399 men who underwent prostatectomy showed that men taking statins were more likely to have organ-confined disease [OR 0.66 95 % CI (0.50–0.85)]. Moreover, the 16 % of men taking statins with a preoperative PSA ≥10 ng/ml [OR 0.35 95 % CI (0.13–0.93), $p=0.02$] were also less likely to have high-grade PC. Patients who used statins for ≥1 year also had lower risk of recurrence compared to nonusers [HR 0.77 95 % CI (0.41–1.42)].

Breau et al. [60], using a population-based group of 2,447 men who were followed from 1990 to 2007, found that the 634 men on statins had a decreased risk of PC diagnosis [HR 0.36 95 % CI (0.25–0.53)] and high-grade PC [HR 0.25 95 % CI (0.11–0.58)]. Patients taking statins were also at decreased risk of undergoing prostate biopsy [HR 0.31 95 % CI (0.24–0.40)]. Furthermore, men using statins for the greatest period of time had the lowest risk of these outcomes (all trend tests $p<0.05$).

Kollmeier and colleagues [70] in a retrospective study examined 1,681 patients treated for PC with radiotherapy between 1995 and 2007, of which 382 subjects were taking a statin. The 5- and 8-year PSA relapse-free survival (PRFS) rates for statin users were 89 and 80 %, in comparison to 83 and 74 % for those not taking a statin ($p=0.002$). In a multivariate analysis, taking a statin was associated with improved PRFS [HR 0.69 95 % CI (0.50–0.97), $p=0.03$]. Moreover, in examining patients diagnosed in a high risk group, Kollmeier et al. demonstrated that statin users exhibited improved PRFS [HR 0.52 95 % CI (0.30–0.91), $p=0.02$] in comparison to those not on a statin. Statin use was not associated with improved metastasis-free survival.

Using a cohort of 55,875 men in the US veterans population who were followed from 1997 to 2007, Farwell et al. [46] reported that statin users were 31 % less likely [HR 0.69 95 % CI (0.52–0.90)] to be diagnosed with PC in comparison with men taking antihypertensive medication. In addition, patients taking statins were 60 % less likely [HR 0.40 95 % CI (0.24–0.65)] to be diagnosed with high-grade PC and 14 % less likely [HR 0.86 95 % CI (0.62–1.20)] to be diagnosed with low-grade PC. This report is notable because the patient cohorts were well controlled for medical attention and access to medical care. A confounding problem in interpreting observational studies is whether there is an extraneous difference between the statin-taking vs. not-taking patient cohorts. One alternative explanation for some of the results is that statin users have greater access to health care or are more invested in their health (more "health seeking"). In these cases, cancer might be diagnosed earlier, resulting in a greater incidence of early stage disease. Because the two groups compared in the Farwell et al. study were plausibly equally health seeking, this confounder may not apply.

Alizadeh et al. [71] analyzed a group which included 381 patients treated with either radiotherapy or brachytherapy for low-risk ($n=152$), intermediate-risk

($n=142$), or high-risk ($n=87$) localized PC. 45.1 % were taking statins, 37.0 % were taking anticoagulants (AC), and 27.6 % were taking both. Exclusive users of statins compared with users of neither drug class had a lower adjusted odds ratio [OR 0.29 95 % CI (0.09–0.88), $p=0.03$] of having a PSA level >20 ng/mL. In addition, concomitant AC and statin use was associated with a reduced likelihood of a PSA level >20 ng/mL and an increased likelihood of a PSA <10 ng/ml. Similarly, exclusive statin use was associated with a greater probability of having a PSA level <10 ng/mL [OR 2.9 95 % CI (1.3–6.8), $p=0.012$]. Although these investigators found no association with low-risk localized PC ($p=0.3$), they reported a significant effect between the concomitant use of statins and ACs with high-risk localized PC [OR 0.43 95 % CI, (0.21–0.87), $p=0.02$].

A study by Marcella et al. [72] including 379 cases and controls in which PC was the cause of death showed in adjusted analysis a decrease in PC deaths among statin users compared to age-matched controls [OR 0.45 95 % CI (0.29–0.71), $p=0.0006$]. Further analysis demonstrated that high-potency statins (cerivastatin, atorvastatin, and simvastatin) were associated with a significant risk reduction [OR 0.27 95 % CI (0.15–0.48), $p<0.0001$], while low-potency statins (pravastatin, lovastatin, and fluvastatin) were associated with a risk reduction that did not reach significance [OR 0.69 95 % CI (0.33–1.45)].

In summary, observational studies of the effects of statin drugs on PC risk, which included substantial numbers of subjects, largely support the hypothesis that statins reduce advanced PC risk.

Recent reports that have focused on PC indicate that prolonged statin therapy may have a chemopreventive effect against aggressive PC, while large randomized trials of statin drugs that report on overall cancer (including PC) do not support this conclusion [73–75]. We have reviewed extensively the literature on randomized trials and have cited several concerns about the all-cancer studies: their relatively brief duration, few PC cases, lack of recording the grade or stage of the cancer, large crossover of patients from control to statin groups (and vice versa) due to usual care requirements or toxicity, and the over-representation of pravastatin, the least potent of the statins, to reveal why these studies cannot be conclusive with respect to statin use and PC risk [36, 63, 76].

Preclinical Studies

The initial evidence that pharmacological reduction of cholesterol levels systemically altered prostate cell growth and/or survival was first reported by Schaffner and colleagues [77]. These investigators demonstrated that they could induce prostate regression in preclinical models by oral administration of hypocholesteremic agents (e.g., the polyene macrolide candicidin) [77] (reviewed in [78]). However, further progress on a role for cholesterol in PC incidence and progression in animal models stalled for a long period until studies by Zhuang et al. [79] and Solomon et al. [80] sparked renewed interest. Using the LNCaP xenograft model, hypercholesterolemia

was shown to increase rates of growth of subcutaneous prostatic tumors, whereas hypocholesterolemia retarded tumor growth. In these studies, hypercholesterolemia was associated with increased tumor cell proliferation, higher levels of activated Akt (a critical kinase in PC progression), as well as increased levels of intratumoral androgen [79–81]. Other groups have used spontaneous PC models and have come to similar conclusions. In the autochthonous TRAMP mouse model [82], hypercholesteremia was shown to result in increased prostate tumor volume, tumor incidence, and metastases to the lung. These tumors exhibited increased proliferation, angiogenesis, expression of cyclin D1, and expression of scavenger receptor class B type 1(SR-B1). Taken together, these reports suggest that circulating cholesterol affects a range of signaling pathways and physiologic mechanisms, findings that have the potential to explain the clinical observations in humans described above.

Cholesterol-Sensitive Mechanisms in Prostate Cancer Progression

There are a number of possible mechanisms by which excess cholesterol could affect responses by malignant cells, including the ability to provoke inflammatory responses as well as having important effects on membrane organization, cell proliferation, and steroidogenesis. All of these processes could be relevant to PC progression. Here we briefly outline the evidence for some of the potential mechanisms.

Inflammation

The pathological consequence of hypercholesterolemia in humans is prominently observed in the formation of atherosclerotic lesions, with elevated LDL levels resulting in the accumulation of LDL particles in the arterial intima, where they are enzymatically modified, becoming inflammatory agonists [83]. Accumulation of these deposits over time induces the inflammatory and pathological changes that are the hallmarks of atherosclerosis. Clinical consequences of atherosclerosis can be reversed 20–40 % by prolonged treatment with statins [84], where risk reduction is proportional to the extent of LDL lowering. These and other data have identified cholesterol, and its synthetic products (e.g., cholesteryl esters), as important mediators of inflammation within the cardiovascular system [83].

About 20 % of human cancers are thought to develop as a consequence of chronic inflammatory or infectious conditions [85]. Increasing evidence now links pathologic or premalignant changes in the prostate, including PC, with inflammation [86]. Human CD4+ and CD8+ T cells recognize antigenic determinants in the PSA protein [87], suggesting that prostatic secretory products, may result in autoimmunity. IL-6 and IL-8, inflammatory mediators, are also mitogens for prostate cells [88, 89], hinting at a potential role for inflammatory components in disrupting prostate tissue homeostasis by altering the balance between cell growth and death.

Foci of epithelial atrophy in human prostate tissues are accompanied by inflammatory infiltrates, a condition described as "proliferative inflammatory atrophy" (PIA) [86]. Transitions between zones of PIA, or proliferative atrophy without inflammatory infiltrate, and high-grade prostatic intraepithelial neoplasia (PIN) have been described [86]. Inflammatory processes preceding neoplastic changes are also noted in preclinical models. For example, neonatal estrogen imprinting of the prostate causes lobe-specific inflammation, hyperplasia, and PIN-like lesions in adult animals [90]. Investigations into PC susceptibility loci have identified a number of genes involved in immunity, including *RNASEL* [91], *MSR1* [92], and *TLR4* [93]. Other loci involved in inflammation have also been associated with increased PC risk [93, 94]. In total, although the data are incomplete, these observations suggest that the prostate is susceptible to several types of inflammatory disruptions that can result in pathology, with cholesterol potentially contributing to sustained inflammation. This also points to the intriguing possibility that the action of statins on PC might be accounted for by the drugs' anti-inflammatory activity [95]. However, cholesterol promotes PC progression in immunodeficient preclinical models, suggesting that inflammation alone is unlikely to explain the PC–cholesterol association [79–81].

Membrane Organization

Experimentation into the biophysical properties of the plasma membrane, of the behavior of glycosylphosphatidylinositol (GPI)-anchored proteins in membranes, of membrane organelles termed caveolae, and of intracellular transport processes provides evidence for the existence of a distinct type of cholesterol rich-membrane microdomain typically referred to as the lipid raft [96–116]. These membrane regions contain high concentrations of cholesterol and fatty acids with long saturated acyl chains (e.g., sphingolipids), relative to other plasma membrane domains. The acyl chain composition of the lipids is a major determinant of lipid segregation into rafts, with cholesterol providing structural order to the lipid bilayer. At high concentrations, the tight packing of these components confers structure onto the liquid-ordered phase, and these membrane patches are physically separable from the liquid-disordered membrane using biochemical methods [117, 118].

Lipid rafts are small heterogeneous membrane domains consisting of cholesterol, sphingolipids, and glycolipids. They variably contain cohorts of (GPI)-anchored proteins, src family kinases, G proteins, and other components [107, 116, 119–127]. Many studies suggest that lipid rafts, as discrete domains within the "lipid sea," serve as privileged sites for certain types of cell signaling, signal pathway cross-talk, and signal amplification [98, 118, 123, 128–136].

Our current understanding of lipid rafts is incomplete, and certain earlier ideas about rafts have given way to subsequent concepts [137–142]. For example, results of single molecule tracking studies have suggested that the large stable raft domains that were once thought possible are unlikely to exist [139, 143, 144]. Consistent

with these findings, we have argued that rafts are small and heterogeneous but are subject to alteration in size and composition as a consequence of specific perturbations (e.g., excess cholesterol) [107, 119–121]. This paradigm fits with even the most critical reports concerning lipid raft form and function and is in fact essential to understanding how excess cholesterol may affect cellular behavior.

Signals vital to PC cell survival and progression are transmitted through rafts. Studies [145–148] have demonstrated that some proteins, which are critical for malignancy, are regulated by lipid rafts and that alteration in membrane cholesterol affects in measurable ways the signals generated by these molecules. A subpopulation of epidermal growth factor receptor (EGFR) residing within PC cell lipid rafts exhibits greater activity and is more highly phosphorylated than the receptor population residing in non-raft membranes. Moreover, signaling by the EGFR to downstream effectors is disrupted by cholesterol targeting [148, 149]. In addition, a subpopulation of the serine–threonine kinase Akt, resident within rafts, phosphorylates a different set of substrates than non-raft Akt. This raft-resident Akt is inhibited by cholesterol level reduction [145]. Signaling by LXRs (liver X receptors) leads to PC cell apoptosis by reducing the level of phosphorylated Akt within rafts, in a process precipitated by LXR-stimulated cholesterol efflux and reversed with the addition of exogenous cholesterol. Taken together these observations suggest that cholesterol regulates lipid raft dynamics, which in turn alters vital signaling pathways, with increased cholesterol acting to protect cells from apoptosis through effects on lipid rafts [150].

Cell Proliferation

Cholesterol has been known for many years to effect the proliferation of animal cells [151–153], with cholesterol synthesis tightly synchronized to cell cycle progression [152]. Research aimed at determining a specific effect of cholesterol on cell cycle transit have shown that reducing cholesterol levels through synthesis-blockades causes cells to growth arrest [154]. Whether low cholesterol leads to growth arrest because the material for membrane synthesis becomes limiting or because of a more specific regulatory role is unclear. However, present observations suggest that cholesterol plays an essential role in cell cycle progression in animal cells. In several species, absence of the principal membrane sterol causes growth arrest despite the availability of sufficient amounts of a related, membrane-compatible sterol (e.g., replacement of cholesterol with ergosterol). In these cases, a small amount of native sterol, insufficient for membrane synthesis, is necessary to restore cell cycle progression [155–158].

The rapid growth and high metabolic rate of malignant cells necessitates large amounts of cholesterol, which is distinct from that involved in regulating cell cycle transit. Interestingly, so much cholesterol may be required to sustain a growing neoplasm that cancer is able to lower serum cholesterol. Examples of this phenomenon include Sherwin et al. who reported that men developing cancer had a

22.7-mg/dL decrease in their cholesterol levels vs. matched survivors [159]; and Keys et al. who found that men who died of cancer within 2 years of the study's initiation had cholesterol levels 9.5 % lower than the average of all men at entry [160]; men dying from cancer 1 year after their final cholesterol measurement had concentrations that were 24–35 mg/dL lower than controls (i.e., those not dying); those succumbing 2–5 years after cholesterol measure had levels 4–5 mg/dL lower than controls; and those succumbing to cancer 6–10 years after cholesterol measure had concentrations 2 mg/dL lower than controls [161]. Cancer may reduce circulating cholesterol levels because of increased rates of metabolism, a phenomenon related to the Warburg effect [162–164], in which an abundance of macromolecules is needed by the tumor cells to support rapid growth. Prior to the advent of the statin era it was thought that hypocholesterolemia was a potential risk factor for cancer, but this notion has since been abandoned [36]. Because of the essential role of cholesterol in tumor cell proliferation, cholesterol lowering may induce apoptosis in PC cells as they progress through the cell cycle [165].

Steroidogenesis

Prostate tumor cells respond to androgens via the androgen receptor (AR), a nuclear receptor, a transcription factor that controls PC cell proliferation in all cancer stages, including castration-resistant disease [166–168]. In the last few years multiple lines of evidence have converged on the hypothesis that PC cells perform intratumoral steroidogenesis (androgen synthesis) [169–171] at sufficient levels to activate the AR, explaining, in part, the development of castration resistance. Androgen-depleted PC cells have the capacity to synthesize dihydrotestosterone (DHT) from acetic acid, revealing that the entire mevalonate–steroidogenic pathway is functionally intact [169]. All enzymes necessary for testosterone and DHT synthesis are present in human primary and metastatic PC [171], implying that de novo steroidogenesis may be an essential mechanism of disease progression in the hormone-repressed state.

Cholesterol is a necessary precursor in androgen synthesis and therefore may promote PC growth through effects on steroidogenesis. To test this possibility we used the in vivo LNCaP PC xenograft mouse model and diet-induced hypo- and hypercholesterolemia to demonstrate that circulating cholesterol levels are significantly associated with both tumor size ($R=0.3957$, $p=0.0049$) as well as intratumoral levels of testosterone ($R=0.41$, $p=0.0023$) [81]. We also demonstrated that the xenograft tumors expressed the full spectrum of steroidogenic enzymes necessary for androgen biosynthesis from cholesterol. Circulating cholesterol concentrations in the mice correlated directly with prostatic tumor expression of CYP17A, an enzyme required for de novo synthesis of androgens from cholesterol ($R=0.4073$, $p=0.025$) [81]. This result suggests that cholesterol acts not only as an essential precursor but also as a pathway agonist, stimulating the upregulation of steroidogenic gene expression.

Conclusions

Epidemiological observations and preclinical models suggest that hypercholesterolemia plays an important role in PC progression, with many human and mechanistic studies now supporting roles for cholesterol in PC progression and for cholesterol-lowering drugs in retarding PC growth. Cholesterol functions as a mediator of cell proliferation, membrane dynamics, inflammation, and steroidogenesis, thus providing multiple avenues for this lipid to contribute to the clinical disease.

At this point the scientific basis for therapeutically targeting cholesterol is robust enough to pursue clinical strategies that exploit the relationship between cholesterol and PC in select patient populations. One such approach would be to recommend enhanced cholesterol lowering for chemoprevention in at-risk populations. It is also possible that in the next decade we will witness the use of cholesterol-lowering regimens as adjuvant therapy to treat existing PC in order to slow progression and/or render the cancer more susceptible to conventional therapies. It may even be possible to more effectively treat CRPC by applying multiple approaches that target steroidogenesis, such as using abiraterone, a CYP17 inhibitor that suppresses androgen synthesis, with a cholesterol-lowering regimen. We suggest that sufficient mechanistic and human data are now available to support the application of these strategies clinically in the case of patients managed by active surveillance and to test the feasibility of using cholesterol-targeting strategies to slow disease progression.

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Part II
Genetics

Chapter 4
PTEN in Prostate Cancer

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Abstract PTEN is one of the most commonly deleted/mutated tumor suppressor genes in human prostate cancer. As a lipid phosphatase and negative regulator of the PI3K/AKT/mTOR pathway, PTEN controls a number of cellular processes, including survival, growth, proliferation, metabolism, migration, and cellular architecture. Over the past 15 years since its discovery, a number of mechanisms governing PTEN expression and function, including transcriptional and post-transcriptional regulation, post-translational modifications, and protein–protein interactions, have been shown to be altered in human prostate cancer. The functions of PTEN within the cell have been expanded to include phosphatase-independent roles and functions within the nucleus. The generation of genetically engineered mouse models (GEMs) with deletion of *Pten* has further revealed that varying degrees of *Pten* loss in combination with other genetic alterations are able to recapitulate all spectrums of human prostate cancer, from tumor initiation to metastasis. With new methods of genomic and transcriptional analysis of human prostate cancer specimens, PTEN loss can potentially be used as a diagnostic and prognostic biomarker for prostate cancer, as well as predict patient responses to emerging PI3K/AKT/mTOR inhibitors. Finally, deeper insight into communication between the PI3K/AKT/mTOR and Ras/MAPK signaling pathways has led to the creation of metastatic murine prostate cancer models that develop lethal metastases, while new understanding of a feedback loop between PTEN and androgen receptor (AR) controlled pathways has unveiled a new mechanism for the development of castration-resistant prostate cancer (CRPC). Our expanded knowledge of PTEN and its role in prostate cancer

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initiation and progression will inform the rational design of novel therapeutics that target PTEN-controlled pathways alone or in combination with other related pathways for the treatment of metastatic and castration-resistant prostate cancer.

Introduction

Although partial or complete loss of chromosome 10 in brain, bladder, and prostate cancers was identified as early as 1984 [1], it was not until 1997 that three independent groups, through mapping of mutations on chromosome 10 and cloning of a novel phosphatase, identified a tumor suppressor gene at the 10q23.31 locus named by different laboratories as the *p*hosphatase and *ten*sin homolog (PTEN), *m*utated in *m*ultiple *a*dvanced *c*ancers 1 (MMAC1), and *T*GF-β-regulated and *e*pithelial cell-enriched *p*hosphatase 1 (TEP1) [2–4]. PTEN is a nonredundant phosphatase that antagonizes the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, one of the most important and well-studied cancer promoting pathways. As PTEN is the only known 3′ phosphatase counteracting the PI3K/AKT pathway, it is not unexpected that loss of PTEN has a significant impact on prostate cancer progression. Indeed, loss of heterozygosity (LOH) of *PTEN* occurs frequently in many advanced stage sporadic tumors, including ~60 % of advanced prostate cancers [2]. Germline PTEN gene mutations account for the majority (80 %) of cases of Cowden Syndrome, an autosomal dominant multiple hamartoma syndrome that leads to an increased propensity for patients to develop breast [5], endometrial [5, 6], and thyroid cancers [7–9]. However, prostate cancer has not been associated with Cowden Syndrome and germline PTEN loss [10, 11], perhaps providing credence to the understanding that loss of PTEN is a late event in prostate carcinogenesis [12, 13].

In this chapter, we will review PTEN structure, function, and regulation. The consequences of loss of PTEN regulation and function in different stages of prostate cancer development, as well as the potential use of PTEN loss as a biomarker for prostate cancer prognosis and prediction of patient responses to PI3K/AKT/mTOR pathway inhibitors, will also be addressed. Due to space limitations, some important topics, such as the role of PTEN in prostate stem cell/cancer stem cell maintenance and crosstalk with the tumor microenvironment, cannot be covered. However, these topics have been covered extensively by several outstanding reviews [14–17].

PTEN Structure and Function

PTEN Structure

The *PTEN* gene comprises nine exons and encodes a protein of 403 amino acids [18]. The amino acid sequence of the PTEN tumor suppressor is considerably homologous to dual-specific protein phosphatases and tensin, a chicken cytoskeletal

protein [2]. The crystal structure of PTEN revealed an expanded active site pocket for binding to its substrates and a C2 domain, which mediates membrane attachment of cell signaling proteins. Three other functional domains have also been identified: a short phosphatidylinositol-4,5-bisphosphate (PIP2) binding domain on the N terminus, and PEST sequences and a PDZ interaction motif on the C-terminal tail that regulate protein stability and binding to PDZ domain-containing proteins, respectively [19]. The binding of PIP2 to PTEN produces a conformational change in the enzyme, leading to allosteric activation [20]. The positive charge of PTEN's substrate binding pocket is also important for accommodating larger acidic substrates such as phosphoinositides. The PTEN phosphatase domain is evolutionarily conserved, and is the recipient of 40 % of its cancer-associated mutations, which occur most commonly through either a C124S mutation that abolishes both lipid and protein phosphatase activity or a G129E mutation that abrogates only its lipid phosphatase activity [21–23]. Although the phosphatase domain is responsible for PTEN's physiological activity, other PTEN tumorigenic mutations occur on the C-terminal C2 domain and tail sequence, highlighting an important role of the C terminus in maintaining PTEN protein stability [24, 25]. The fact that tumor-associated mutations occur in all PTEN functional domains indicates that each of these regions is biologically relevant to PTEN function. In prostate cancer, PTEN loss most commonly results from a somatic mutation generated through copy number loss rather than point mutation [26], although recent exome sequencing has identified several recurrent mutations in the PTEN gene [27, 28].

PTEN and Regulation of the PI3K/AKT/mTOR Pathway

PI3K/AKT signaling plays a critical role in regulating growth responses, homeostasis, and longevity. At the cellular level, the PI3K/AKT pathway controls cell growth, migration, differentiation, and survival. Activation of the PI3K/AKT pathway is also frequently detected in human cancers [29]. PTEN is a unique lipid phosphatase that removes the phosphate from the D3 position of phosphatidylinositol-3,4,5-triphosphate (PIP3), a product of PI3K, thus directly antagonizing the action of PI3K [23, 30, 31]. PIP3 accumulation at the plasma membrane through PI3K activity results in recruitment and activation of important kinases involved in cell growth and survival, including phosphoinositide-dependent kinase-1 (PDK1) and AKT family members, via their pleckstrin homology (PH) domains [23, 30, 31]. In this manner, PTEN negatively regulates the PI3K/AKT pathway by inhibiting downstream AKT activation.

AKT isoforms (AKT1, AKT2, AKT3) are activated by phosphorylation at two different residues: Thr308 by PDK1 [32] and Ser473 by mammalian target of rapamycin complex 2 (mTORC2) [33]. Activated AKT drives cell survival, proliferation, growth, angiogenesis, and metabolism by phosphorylating downstream signaling proteins, which include inhibitory phosphorylation of GSK3β, FOXO, BAD, p21, p27, and PGC1, and activating phosphorylation of mammalian target of

rapamycin complex 1 (mTORC1), IKK-β, MDM2, ENTPD5, SREBP1C, AS160, and SKP2 [33, 34]. AKT promotes cell cycle progression and proliferation by directly inhibiting p21 and p27 and alleviating GSK3β-induced cyclin D1 degradation. Moreover, inhibition of GSK3β has been shown to prevent the degradation of β-catenin, which can further stabilize cyclin D1 mRNA and promote G_1-phase/S-phase progression [34, 35]. Activation of AKT also helps evade apoptosis directly by phosphorylation of the pro-apoptotic protein BAD [36]. In this regard, it is not surprising that re-expression of WT PTEN in *PTEN* null prostate cancer cell lines leads to apoptosis [37].

AKT directly activates the mTOR pathway by phosphorylating TSC2, which dismantles the TSC1/TSC2 complex that normally inhibits Rheb. Rheb, now free from TSC1/TSC2 inhibition, can stimulate the phosphotransferase activity of mTORC1 [38]. AKT may also activate mTORC1 by phosphorylating and inhibiting PRAS40, a negative regulatory subunit of the mTORC1 complex [33, 39]. Active mTORC1 phosphorylates p70 ribosomal protein S6 kinase (S6K) and 4E-binding protein (4EBP1), which in turn initiates cap-dependent protein translation [40]. Therefore, as a consequence of PTEN inactivation, PI3K/AKT/mTOR pathway activation leads to enhanced translation of mRNAs involved in protein synthesis, cell growth, and proliferation.

Interestingly, mTORC1 signaling also triggers a negative feedback loop that inhibits the PI3K/AKT pathway. This occurs through the phosphorylation and degradation of insulin receptor substrate 1 (IRS1), a crucial effector of insulin signaling, by S6K [41, 42]. Conversely, inhibition of mTORC1 results in hyperactivation of the PI3K/AKT pathway, as well as increased signaling through the Ras/MAPK pathway. The growth factor receptor GRB10 is a novel mTORC1 substrate that mediates feedback inhibition of the PI3K/AKT and Ras/MAPK pathways by direct inhibition of IRS proteins [43, 44]. In contrast, PTEN loss can reverse mTORC1-mediated negative feedback inhibition of the PI3K/AKT/mTOR pathway by activating both the upstream and downstream arms of the PI3K/AKT/mTOR pathway. Therefore, effective inhibition of tumors with PTEN loss will require inhibition of both mTORC1 and other signaling molecules upstream in the pathway, including PI3K and AKT.

PTEN and Metabolism

Recent studies have suggested that metabolic reprogramming is a requirement for the rapid cell proliferation of cancer cells. As opposed to differentiated and nonproliferating cells, which primarily utilize mitochondrial oxidative phosphorylation to generate the ATP needed for cellular processes, rapidly proliferating cells, including stem cells and cancer cells, tend to convert most glucose to lactate, even in the presence of oxygen, through aerobic glycolysis and a phenomenon known as the Warburg effect [45]. In this way, cancer cells exhibit high rates of glycolysis with increased glucose and glutamine uptake and lactate production, as well as increased

biosynthesis of lipids, amino acids, and nucleic acids, macromolecules that are needed to compensate for anabolic growth [46].

The PI3K/AKT pathway plays a key role in the regulation of glucose metabolism given its position downstream of the insulin receptor. The PI3K/AKT pathway enhances insulin-mediated glucose uptake and membrane translocation of the glucose transporter GLUT1, which has been positively correlated with higher tumor grades and Gleason scores [47], by way of mTORC1 activation and cap-dependent translation [48], and GLUT4, by way of inhibition of AS160 [49]. As PI3K/AKT signaling leads to increased production of HIF1α [50, 51], a transcription factor that regulates the transcription of the *Glut-1* gene [52], it is likely that both the PI3K/AKT pathway and HIF1α activation contribute to higher levels of GLUT1 and enhanced glucose uptake [53]. Increased HIF1α expression also upregulates expression of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis that may further promote tumor metabolism by facilitating access to nutrients in the blood [54]. Conversely, stimulation of the PI3K/AKT pathway blocks gluconeogenesis by preventing both FOXO and PGC1α activation [55, 56]. AKT may indirectly activate glycolysis as well by directly phosphorylating PKF2, whose product, Fru-1,6-P2, is a potent allosteric activator of the glycolysis rate-controlling enzyme PFK1 [32, 57]. A recent study using siRNA-mediated gene silencing in metastatic prostate cancer cell lines revealed that PFKFB4, an isoform of PFK2 that is required for glycolysis, is essential for survival of prostate tumor cells and that ablation of PFKFB4 inhibits tumor growth in a xenograft model [58].

In comparison to other epithelial cancers, primary prostate cancers are less glycolytic and, therefore, not sensitive to FDG-PET imaging until reaching the metastatic stage [59, 60]. On the other hand, prostate cancer is known to be lipogenic, and C-11-acetate and F18-choline have been used, although in limited scale, in prostate cancer imaging [59, 61]. Recent studies suggest that the PI3K/AKT pathway can regulate lipid metabolism as well to further promote anabolic growth through the Warburg effect. Upon PTEN loss and through inhibition of GSK3, the PI3K/AKT axis activates the transcription factor SREBP1C, which in turn transcribes genes involved in cholesterol and fatty acid biosynthesis [62, 63]. PTEN has also been shown to regulate the synthesis of long chain saturated fatty acids by inducing the downregulation of fatty acid synthase (FAS), a lipogenic enzyme overexpressed in many human cancers, including prostate cancer, in a lipid phosphatase-dependent manner [64]. Therefore, PTEN loss in prostate cancer cells may increase FAS protein expression, which is elevated in tumors with a poor prognosis [65]. Collectively, these data indicate that both upstream and downstream components of the PTEN regulated PI3K/AKT/mTOR pathway are involved in the metabolic reprogramming required to sustain the rapid growth and proliferation of tumor cells by (1) increasing glucose metabolism via aerobic glycolysis and (2) promoting macromolecule biosynthesis via lipogenesis.

The recent creation of a mouse model with global PTEN overexpression, the "Super-PTEN" model, has demonstrated that PTEN elevation at the organism level results in diminished glucose and glutamine uptake and increased mitochondrial

oxidative phosphorylation, resulting in a reversion to a more healthy metabolism [66]. PTEN elevation in this model coordinates this metabolic shift by negatively regulating both PI3K/AKT-dependent pathways, such as mTORC1 activation of PKM2, a controller of glycolytic flux [67], and PI3K/AKT-independent pathways, such as degradation of PFKFB3, a key regulator of glycolysis [68], through APC/Cdh1 activation [66]. Interestingly, these "Super-PTEN" mutants are resistant to oncogenic transformation, demonstrating that inhibition of the metabolic reprogramming to aerobic glycolysis through PTEN expression or inactivation of the PI3K/AKT pathway may be sufficient to obstruct tumor propagation [66]. These outcomes suggest that PTEN overexpression may indeed be an attractive option for cancer prevention and therapy.

PTEN in the Nucleus

It was initially assumed that PTEN is exclusively localized in the cytoplasm. However, following the discovery that PTEN contains dual nuclear localization signal-like sequences [69], it has been well recognized that PTEN can localize to the nucleus, and recent studies have illustrated the important functions of nuclear PTEN in regulating cell cycle progression and genomic integrity. Indeed, not only is there a marked reduction in nuclear PTEN in rapidly cycling cancer cell lines in comparison to resting or differentiated cells [70–73], but absence of nuclear PTEN has also been associated with reduced overall survival in prostate cancer patients [74].

Oxidative stress is one of the physiological stimuli that regulate the accumulation of nuclear PTEN [75]. Oxidative stress inhibits PTEN nuclear export, a process dependent on phosphorylation at Ser380. Nuclear PTEN, independent of its phosphatase activity, can regulate p53 stability and transcriptional activity [76, 77], leading to p53-mediated G_1 growth arrest, cell death, and reduction of reactive oxygen species (ROS) production [75]. Nuclear PTEN is also sufficient to reduce human prostate cancer xenograft growth in vivo in a p53-dependent manner [75], suggesting a unique role of nuclear PTEN to arrest and protect cells following oxidative damage and to regulate prostate cancer development.

Nuclear PIP3, unlike cytoplasmic PIP3, is insensitive to the lipid phosphatase activity of PTEN, implying nuclear functions for PTEN beyond its role as a negative regulator of the PI3K/AKT pathway [78]. This, however, is at odds with another finding that forced nuclear expression of PTEN can reduce nuclear levels of P-AKT, although it was not demonstrated whether this mechanism occurred through a PI3K-dependent or -independent pathway [79]. One proposed function of PTEN in the nucleus is to induce G_1 cell cycle arrest in part by reducing cyclin D1 levels through its protein phosphatase activity [80] or through controlling MAPK signaling [81]. Nuclear PTEN maintains chromosomal stability by physically associating with centromeres through docking onto CENP-C, a centromeric-binding protein [82]. Moreover, nuclear PTEN, through a phosphatase-independent mechanism, enhances DNA repair through increasing the activity of RAD51, a protein implicated in

double strand break (DSB) repair [82]. Not surprisingly, PTEN-null cells develop spontaneous DNA DSBs at a high rate [82]. Cytoplasmic PTEN can also contribute to DSB repair by inhibiting AKT-dependent sequestration of the cell cycle regulator CHK1 in the cytoplasm [83]. In this fashion, PTEN helps to maintain the G_2/S cell cycle checkpoint and likewise prevents genomic instability and DSBs. As PTEN loss leads to homologous recombination defects in human tumor cells through downregulation of RAD51 and CHK1 in the nucleus, tumor cells display increased sensitivity to inhibitors of PARP [84–86]. These findings provide evidence for the use of PARP inhibitors in patients with PTEN-deficient prostate cancer. However, a recent study of clinical prostate cancer specimens suggests that PTEN loss is not associated with reduced RAD51 mRNA or protein expression in primary prostate cancer, and that PTEN-deficient cells only exhibit mild sensitivity to PARP inhibition, casting doubt on whether PTEN is a useful biomarker for response to PARP inhibitors in prostate cancer [87].

Nuclear PTEN directly increases the antitumor and E3 ligase activity of APC/C through a phosphatase-independent mechanism by promoting the association of APC/C with its activator CDH1 [88]. The APC/C-CDH1 complex contains tumor suppressive activities that degrade oncogenic proteins such as PLK1 and Aurora kinases [89, 90]. In this regard, combining PLK and Aurora kinase inhibitors with PI3K/AKT inhibitors may provide increased efficacy in treating PTEN-deficient prostate cancer. Altogether, these findings suggest that the tumor suppressive functions of PTEN are in part due to its functions within the nucleus. New insights into the regulation of PTEN subcellular localization and the functions of PTEN in the nucleus may shed light on novel biomarkers and therapeutics for the treatment of prostate cancer.

PI3K/AKT/mTOR-Independent Functions of PTEN

Although most phenotypes associated with PTEN loss can be accounted for by the activation of the PI3K/AKT pathway, transgenic models with prostate-specific overexpression of p110β or a constitutively active form of AKT develop only localized, precancerous PIN lesions, suggesting that PTEN possesses other tumor suppressor functions independent of the PI3K/AKT pathway [91–93]. Similarly, while conditional deletion of *p110β* or *Rictor*, in addition to *Pten* conditional deletion, prevents the progression of tumor development from PIN to adenocarcinoma, they do not completely prevent prostate cancer initiation [94, 95].

One example of a PI3K/AKT/mTOR-independent mechanism of PTEN regulation is the interaction between PTEN and p53 [96]. PTEN inactivation is known to increase the expression [97] and activation of the p53 repressor MDM2 [98] by a PI3K/AKT-dependent pathway [99] and upregulate p53 through translational mechanisms mediated by mTORC1 [100, 101]. The PTEN C2 domain, which lacks phosphatase activity, can also regulate cell motility [102] and, interestingly, can interact directly with p53 in a phosphatase-independent manner to enhance p53-mediated

cell cycle arrest and apoptosis by promoting the stabilization, acetylation, and tetramerization of p53 [75, 76, 103]. Conversely, p53 can also regulate PTEN at the transcription level [104]. In the *Pten*-null mouse model, deletion of p53 accelerates *Pten*-null prostate cancer by reducing cellular senescence [105]. Concomitant mutations of PTEN and p53 have been detected within individual human tumors, supporting a selective advantage for combined inactivation of both tumor suppressors. However, whether the cooperation of PTEN and p53 loss in overriding cellular senescence promotes human prostate cancer progression needs to be further investigated.

PTEN can also regulate the expression of other tumor suppressors whose functions are commonly lost as an early event in prostate cancer initiation, such as Nkx3.1 [106]. Not only is Nkx3.1 expression downregulated in the PTEN-null murine prostate cancer model, but forced expression of Nkx3.1 in the PTEN-null prostate epithelium prevents prostate cancer initiation and progression [106]. Moreover, while a transcriptional profiling study has indicated that the JNK pathway is activated following PTEN loss in an AKT-independent manner [107], a recent report elucidated that JNK deficiency collaborates with PTEN loss in promoting CRPC [108].

Though the lipid phosphatase activity of PTEN is central to its role as a tumor suppressor, other, phosphatase-independent functions of PTEN are also important. PTEN is a dual-specificity protein phosphatase with activity toward acidic substrates. PTEN is capable of dephosphorylating phosphorylated serine, threonine, and tyrosine residues on peptide substrates in vitro [109], as well as protein substrates such as FAK [110], CREB [111], eIF2 [112], and SRC [113] in vivo, thereby directly inhibiting cell survival, proliferation, and migration. The activation of these PI3K/AKT-independent pathways after PTEN loss suggests that combining PI3K/AKT inhibitors with inhibitors of these other pathways may improve efficacy in treating patients with PTEN-deficient prostate cancer.

PTEN Regulation

Genetic Regulation

Germline PTEN mutations do not predispose men to prostate cancer [10, 11]. However, the 10q23 gene locus is a frequent target for somatic heterozygous deletion in primary, and, more frequently, in metastatic prostate tumors, where loss of heterozygosity (LOH) is found in 20–60 % of tumors [114]. However, the finding that the rate of PTEN LOH and mutations are far less frequent than the detected rate of PTEN loss at the protein level suggests that other, nongenomic alterations may occur that inactivate the second PTEN allele.

Epigenetic Regulation by DNA Methylation

Supporting its important physiological functions, PTEN is constitutively expressed in normal tissues, including infant and adult human prostates. However, PTEN expression can be downregulated on many levels in various physiological settings. Epigenetic inactivation of the PTEN promoter has been described in prostate cancer xenografts, where loss of PTEN protein is a result of promoter methylation [115]; however, this has yet to be shown in primary prostate cancer specimens. Additionally, the zinc-finger transcription factor SALL4 represses PTEN transcription in embryonic stem cells by recruiting an epigenetic repressor complex called the Mi-2/NuRD complex to the PTEN locus [116]. Despite these discoveries, epigenetic silencing of PTEN in prostate cancer has not been demonstrated in the in vivo and clinical setting.

Transcriptional Regulation

Suppression of *PTEN* transcription may have an important and understated role in prostate cancer initiation and progression. PTEN was originally cloned as a gene transcriptionally regulated by transforming growth factor β (TGFβ) [4], which both suppresses and induces PTEN expression depending on the activation status of the Ras/MAPK pathway. When Ras/MAPK is activated, as is common in aggressive, late stage disease, TGFβ suppresses PTEN expression through a Smad4-independent pathway [117]. Alternatively, when the Ras/MAPK pathway is blocked, TGFβ induces PTEN expression through its canonical Smad-dependent pathway [118]. The Ras/MAPK pathway also suppresses PTEN levels through the transcription factor c-Jun [119]. Moreover, the MEK–JNK pathway suppresses PTEN transcription via activation of NF-kB, which directly binds to and suppresses the PTEN promoter [120]. Expression of PTEN is also negatively regulated by the epithelial-to-mesenchymal transition (EMT) transcription factor SNAIL, which is itself activated by PI3K/AKT and RAS/MAPK pathways [121–123]. SNAIL competes for binding to the PTEN promoter with p53, which is a transcriptional activator of PTEN and leads to activation of PTEN transcription during p53-mediated apoptosis [96]. Activated NOTCH1 both positively and negatively regulates PTEN expression through MYC and CBF1, respectively [124, 125]. PTEN transcription can also be upregulated through several other transcription factors, including PPARγ [126] and EGR1 [127], as well as downregulated by BMI1 [128], which regulates prostate stem cell self-renewal and malignant transformation [129]. All in all, transcriptional control of PTEN lies within a network of tumor suppressors and oncogenes controlling various signaling and development programs within normal and cancerous prostate cells.

Post-transcriptional Regulation

PTEN mRNA is also post-transcriptionally regulated by PTEN-targeting microRNAs (miRNAs), a class of endogenous 20–25 nucleotide noncoding RNAs that repress mRNA translation through imperfect base pairing between the seed sequence of the miRNA and the complementary seed match sequence in the 3′ untranslated region of the target mRNA [130]. A number of miRNAs have been reported to promote tumorigenesis by downregulating PTEN expression. For example, miR-22 and the miR-106b-25 cluster, both PTEN targeting miRNA loci, are aberrantly overexpressed in human prostate cancer and are capable of initiating prostate tumorigenesis in vitro and in vivo [131]. The identification of these and other prospective PTEN-targeting miRNAs in serum of prostate cancer patients may be valuable as surrogate markers for PTEN status, and hence could correlate with both disease progression and the potential efficacy of PI3K/AKT inhibitor treatment.

In a newly emerging field of research, the PTEN pseudogene 1 (PTENP1) was found to influence PTEN expression through a coding-independent function, uncovering a new mechanism of gene regulation [132]. Since the PTENP1 mRNA transcript shares vast homology with PTEN mRNA, PTENP1 acts as a decoy for PTEN-targeting miRNAs and can thereby sequester and inhibit the negative regulatory effects of miRNAs on PTEN expression [14]. PTENP1 can, therefore, be considered a competing endogenous RNA or ceRNA. Recent research has uncovered a large network of ceRNA transcripts in prostate cancer that can control PTEN expression by blocking the action of PTEN-targeting miRNAs. These discoveries fortify the existence of a large and complex PTEN tumor suppressor network that can be regulated by coding and noncoding RNAs and can be used to explain the observance of partial or incomplete PTEN inactivation in human prostate cancer [133–135].

Post-translational Regulation

PTEN stability is regulated by various post-translational modifications. When inactivated, PTEN is phosphorylated at various serine and threonine residues on its C-terminal tail, which, in turn, increases PTEN stability [136–138]. This C-terminal phosphorylation results in a more stable yet “closed” state of PTEN, which reduces its plasma membrane localization [139] and its ability to form a complex with PDZ domain-containing proteins [138], thereby reducing its PIP3 lipid phosphatase activity [140–142]. As PTEN is activated, dephosphorylation of its C-terminal tail opens its phosphatase domain, increasing PTEN activity and enhancing its interactions with binding partners, but in turn making PTEN increasingly unstable [143]. Also located in the C-terminal tail, Ser370 can be phosphorylated by a downstream effector of SRC, CK2 [144], while Thr366 appears to be phosphorylated by GSK3β [145]; however, the function of phosphorylation at these sites still remains unclear [146].

The targeting of PTEN to the plasma membrane can also be orchestrated through phosphorylation at Ser 229 and Thr321 on its C2 domain by the protein kinase ROCK [147–149]. Tyr336 of PTEN can also be phosphorylated by RAK, which can act as a tumor suppressor in its own right by regulating PTEN stability and function [150]. Future research may unveil other known and unknown kinases that are capable of phosphorylating PTEN and thereby regulate specific PTEN functions.

The open state of PTEN is also more prone to ubiquitin-mediated proteasomal degradation. Lys13 and Lys289 are conserved sites for PTEN ubiquitination, and monoubiquitination is necessary for the movement of PTEN from the cytoplasm to the nucleus [79]. NEDD4-1 is a recently identified E3 ligase of PTEN that induces both PTEN mono- and poly-ubiquitination [151]. However, NEDD4-1 knockout mice contain no differences in the expression level and subcellular localization of PTEN, hinting that other E3 ligases may be involved in the regulation and localization of PTEN [152]. Along these lines, two other E3 ligases, XIAP and WWP2, have been proposed to mediate PTEN ubiquitination [153, 154].

Similar to other phosphatases, the cysteine residues in the bottom of the PTEN catalytic pocket are very sensitive to oxidation [155]. The catalytic activity of PTEN is attenuated by reactive oxygen species (ROS) through the development of a disulphide bond between Cys71 and Cys124 that is induced during oxidative stress [156, 157]. Furthermore, PTEN can also be acetylated at Lys125 through Lys128 by PCAF and at Lys402 by CBP, inhibiting its catalytic activity while facilitating interactions with PDZ domain-containing proteins [158]. Finally, other forms of PTEN redox regulation have been suggested by research demonstrating the inactivation of PTEN through nitrosylation of cysteine residues in its phosphatase domain [159]. Together, these findings highlight the potential to manipulate mechanisms of PTEN post-translational modifications for use as therapeutics to enhance the tumor suppressive functions of PTEN.

Protein–Protein Interactions

A number of PTEN interacting proteins regulate the tumor suppressive abilities of PTEN by altering its conformation, stability, and subcellular localization. PTEN contains a 3 amino acid C-terminal region that binds to PDZ domain-containing proteins, and these PDZ domains are involved in multiprotein complex assembly [137, 160]. Indeed, the PDZ domain of PTEN mediates interactions with NHREF, which binds to and recruits PTEN to PDGFR to inhibit the activation of the PI3K–AKT pathway [161]. The PTEN PDZ-binding domain binds to several other proteins, including MAGI-2 and MAST205, which appear to enhance the stabilization of PTEN [160, 162, 163]. As PTEN can be found in high molecular mass complexes through size-exclusion chromatography, it was hypothesized that the PDZ-binding domain may be required for such complex formation [164]. However, mutagenesis studies demonstrated that neither PTEN's catalytic activity nor its PDZ binding domain are absolutely required for its complex formation. Instead, PTEN

phosphorylation status has a significant role in its complex assembly [165]. Using two-dimensional gel electrophoresis and mass spectrometry analysis, hnRNPC was identified as a novel PTEN-interacting protein [165]. Indeed, PTEN and hnRNPC are colocalized in the nucleus and may be involved in RNA regulation [165, 166].

Additional proteins are capable of binding to other domains on the PTEN protein. PICT-1 interacts with PTEN by binding to and promoting phosphorylation of the C-terminal tail, conferring PTEN stabilization [167]. Through a yeast two-hybrid screen, β-arrestin was identified as a PTEN-binding partner, binding to PTEN's C2 domain [168]. When PTEN is dephosphorylated at Thr383, this increases the binding affinity of β-arrestin to PTEN, which in turn allows PTEN to negatively regulate cell proliferation through its lipid phosphatase activity, as well as enhance cell migration by reversing the inhibitory effect of the C2 domain [168]. Furthermore, PTEN can directly interact with the regulatory subunit of PI3K, p85, which increases its lipid phosphatase activity and subsequent capability of downregulating the PI3K–AKT pathway [141, 169]. Therefore, p85 can regulate the PI3K/AKT pathway by both negatively regulating PI3K through direct binding to its catalytic subunit, p110, and by positively regulating PTEN activity. Under oxidative stress conditions, DJ-1, which was identified in Drosophila melanogaster, can also directly bind to PTEN, an action that is associated with increased P-AKT levels [170, 171]. Recent screens have identified novel PTEN regulators, including PREX2a [172] and SIPL1 [173], which bind to PTEN directly and inhibit its phosphatase activity against PIP3. MAN2C1 also binds to PTEN and inhibits its function in both prostate cancer cell lines and primary human prostate tumors [174]. Intriguingly, one study found that, of 60 % of primary human prostate tumors that were PTEN-positive, 80 % displayed overexpression of MAN2C1, uncovering a possible new mechanism of PTEN downregulation without genomic loss of PTEN [174]. Despite the discovery of various PTEN protein-binding partners, further investigation is necessary to understand the physiological and clinical relevance of these interactions.

Subcellular Localization

The function of PTEN is also regulated by its subcellular localization. At the plasma membrane, PTEN can regulate directional chemotaxis. PTEN recruitment to the plasma membrane relies on electrostatic interactions with acidic lipids in the membrane, such as phosphatidylserine, PIP2, PIP3, and phosphatidic acid [175], as well as additional protein–protein interactions [143, 176]. PTEN interacts with several membrane-anchored proteins in its dephosphorylated form, including MAGI-2 [160], MAST205 [162], hDLG [138], MVP [177], and PDGFR and NHERF [161], which are thought to be potentially part of a larger PTEN complex, via its C-terminal PDZ domain. NEP has been shown to recruit PTEN to the plasma membrane, which in turn enhances its catalytic activity and subsequently hinders AKT activity [178]. Similarly, the motor protein myosin V regulates the migration of PTEN to the membrane by directly binding to PTEN [179].

PTEN is predominantly localized to the nucleus in differentiated and resting cells in comparison to rapidly cycling cancer cells [72]. The nuclear localization of PTEN is also dependent upon the cell cycle stage, with nuclear PTEN levels highest at the G_1 phase and lowest at the S phase [71]. While some studies have shown that PTEN nuclear localization is dependent upon noncanonical nuclear localization sequences on PTEN and major vault protein-mediated nuclear transport [69], others have shown that nuclear localization of PTEN occurs through passive diffusion through the nuclear membrane [180]. It has been further suggested that PTEN contains a type of cytoplasmic localization signal (CLS) in its N-terminal region that, when mutated, induces the nuclear import of PTEN [181]. Other so-called nuclear exclusion motifs and NLS sequences have been identified that control PTEN localization through a RAN-dependent mechanism [182]; however, how they regulate the shuttling of PTEN between the nucleus and the cytoplasm is not understood. More conclusively, PTEN monoubiquitination by the E3 ligase NEDD4-1 induces the nuclear localization of PTEN [151], while the deubiquitinase HAUSP controls PTEN deubiquitination and nuclear exclusion [183]. Oxidative stress induces the accumulation of PTEN in the nucleus, where it associates with p53 to trigger cell cycle arrest and reduce ROS [75]. Nevertheless, mechanisms involved in the nuclear export of PTEN are still waiting to be uncovered. The use of models utilizing PTEN proteins with mutations that disrupt PTEN localization but maintain PTEN phosphatase activity may provide new understandings into the role of nuclear PTEN.

PTEN Loss as a Biomarker for Human Prostate Cancer

Despite recent and past findings firmly establishing loss of *PTEN* as one of the most common somatic genetic alterations in prostate cancer, prostate cancer specimens are not routinely screened for PTEN loss in the clinical setting. Fluorescence in situ hybridization (FISH) has been used to identify genomic *PTEN* loss, which is found in 9–23 % of high-grade prostatic intraepithelial neoplasia (PIN) lesions [184, 185] and 10–70 % of prostate cancers [12, 13, 186–190], and is correlated with an overall poor prognosis [26, 74, 190–193]. Loss of PTEN expression in the cytoplasm as well as in the nucleus, as determined by FISH and immunohistochemistry (IHC) analysis, is independently associated with decreased disease-specific survival [74, 194]. Part of the reason for these variations may be due to the subjective nature and tediousness of counting the number of fluorescent signals and positive antibody stains relative to control signals and stains to quantify PTEN expression. FISH analysis also lacks the sensitivity to identify minor mutations/perturbations in the PTEN gene locus, as well as other epigenetic and post-transcriptional changes that may influence PTEN expression [13, 195]. Moreover, current research, through the use of “break apart” FISH technology, has revealed that gross chromosomal rearrangements of the PTEN locus occur in prostate cancer, which could very well explain the absence of PTEN expression in tumors designated as harboring genomic loss of only one PTEN allele using conventional single probe FISH [196].

Despite discrepancies in reported rates of genomic *PTEN* loss, a general finding of these studies is that loss of one *PTEN* allele is significantly more frequent than loss of both *PTEN* alleles, although homozygous deletions are associated with advanced disease and metastasis [197, 198]. Haploinsufficiency for PTEN, as well as inactivation of the second *PTEN* allele through nongenomic alterations, may explain why heterozygous *PTEN* deletions outnumber homozygous *PTEN* deletions in human prostate cancer and also result in poor outcomes [184, 190, 199]. Indeed, nearly 70 % of primary human prostate tumors do not contain inactivation of both copies of *PTEN* [105]. In terms of disease progression, the frequency of *PTEN* loss is higher in surgical cohorts enriched for high Gleason grades and aggressive disease stages [192]. *PTEN* loss is more common in hormone refractory and metastatic prostate cancer than in hormone-dependent primary tumors, with homozygous *PTEN* loss in 10 and 50 % of hormone-dependent and metastatic/hormone refractory cases, respectively [74, 184, 185, 189–191, 193, 200, 201]. Therefore, PTEN could serve as a prognostic marker for hormone refractory and metastatic disease. *PTEN* genomic loss is also associated with the TMPRSS2–ERG fusion [26, 185], and recent reports have concluded that these events cooperate to stratify patients with a poorer prognosis in the clinic [192]. A close association between PTEN loss and therapeutic resistance, as demonstrated by a decreased time to biochemical recurrence after radical prostatectomy, adjuvant docetaxel treatment, and radiotherapy, has also been observed [74, 190, 191, 202, 203].

The possibility that FISH and other genomic analyses may fail to detect some cases of PTEN inactivation calls for alternative methods to detect PTEN loss. Considering the role of post-transcriptional and post-translational modifications in PTEN protein expression and subcellular localization as discussed above, quantification of PTEN protein levels using immunohistochemistry (IHC) may be a better indicator of PTEN expression. In a recent study using a rabbit monoclonal antibody against PTEN for IHC analysis, PTEN protein loss was detected in 75–86 % of samples with genomic PTEN loss and was even discovered at times in the absence of genomic *PTEN* loss [194]. Interestingly, 45 and 37 % of tumors with PTEN protein loss did not show genomic deletions measureable by FISH or SNP microarray analysis, respectively, further suggesting that alternative mechanisms of PTEN inactivation exist beyond the genomic level [194]. Moreover, IHC analysis has correlated PTEN protein loss with high Gleason scores, as well as decreased time to metastasis in a cohort of patients having undergone surgical resection [194]. Other studies using large prostate cancer cohorts combining genomic analysis, through comparative genomic hybridization (CGH) and whole-exome sequencing, and transcriptosome analysis have uncovered frequent alterations of the PI3K/AKT pathway in prostate cancer [26–28], which correlated with 42 and 100 % of human primary and metastatic prostate cancer, respectively, as well as high-risk disease [26]. Using network component analysis, 20 transcription factors have been identified whose activities, as deduced from their target gene expression, are immediately altered upon the re-expression of PTEN in a PTEN-inducible system [204]. Notably, the activity of these transcription factors can be used to predict PTEN functional status in human prostate, breast, and brain tumor samples with increased reliability when compared to basic expression-based analysis [204]. With improved mechanisms for detecting

PTEN functional status, PTEN loss could be used not only as a prognostic biomarker for men with prostate cancer but also as a potential predictive marker to identify patients who could benefit from emerging PI3K/AKT pathway therapies.

PTEN in Prostate Cancer Initiation, Progression, CPRC, and Metastasis

Mouse Models of Prostate Cancer

Prostate cancer research has been limited, in part, by the lack of animal models that develop spontaneous prostate tumors in a manner that mimics human prostate cancer. Mouse xenograft models reconstituted from primary human metastatic prostate cancer cells and cell lines have been developed and used extensively in research as preclinical models. However, these xenograft models cannot be used for studying the underlying mechanisms involved in prostate cancer initiation and progression since they are derived from late stage disease. Moreover, many of the key features of the disease, especially the resident tumor microenvironment and the stromal and immune cells that occupy it, are lacking in this immune incompetent system and, therefore, engrafted tumors cannot recapitulate the whole spectrum of human prostate cancer [205]. Genetically engineered mouse (GEM) models of prostate cancer have advanced significantly over the past decade [206–210], and the strong implication of PTEN loss in prostate cancer progression in humans has prompted the expansion of GEM models based on PTEN inactivation. Greater knowledge of the role of PTEN loss as an individual and cooperative agent in prostate cancer development, including initiation, progression and invasion, castration-resistant prostate cancer (CRPC), and metastasis, has been uncovered using mouse models that recapitulate the human disease through genetic loss of the murine homolog of the *Pten* gene (Fig. 4.1).

PTEN Dosage in Mouse Models of Prostate Cancer

PTEN dosage appears to be an important determinant in the development of many epithelial cancers, as demonstrated in various mouse models of *Pten* loss [211]. In the prostate, a hypomorphic PTEN allele, which leads to ~20 % reduction of PTEN levels, shows no sign of neoplastic lesions in the prostate epithelium, while conditional or conventional deletion of one Pten allele causes a 50 % reduction of PTEN levels and leads to precancerous PIN lesions but not cancer, indicating that inactivation of one allele of *Pten* is sufficient to initiate tumorigenesis but not tumor progression [76, 212–216]. Interestingly, by combining a hypomorphic allele with a knockout allele, and thereby reducing PTEN levels by 70–80 %, these mice progress to invasive adenocarcinoma of the prostate [214], indicating that a more

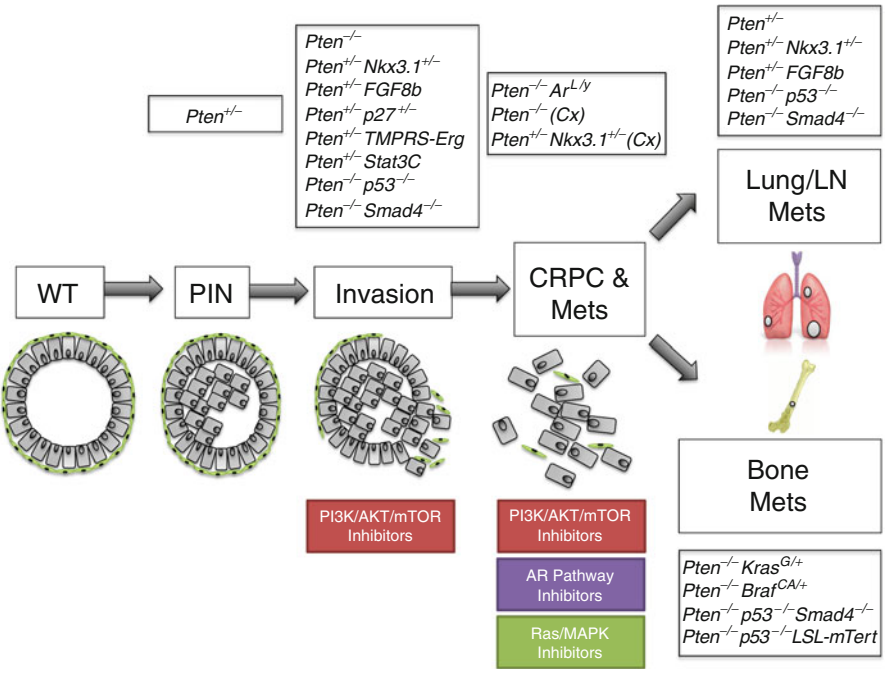

Fig. 4.1 *Pten knockout mouse models of prostate cancer. Pten* heterozygous (*Pten*$^{+/-}$) or homozygous (Pten$^{-/-}$) loss, alone or in combination with other pathway alterations, is able to recapitulate all stages of human prostate cancer, including initiation (PIN), cancer progression/invasion, CRPC, and metastasis. Studies in these murine models provide credence for the use of PI3K/AKT/mTOR, AR, and Ras/MAPK inhibitors for the treatment of metastatic CRPC. In the figure, *gray squares* represent luminal cells, while *green ovals* represent basal cells. *Cx* castration, *PIN* prostatic intraepithelial neoplasia, *LN* lymph nodes, *Mets* metastases, *WT* wild type, *CRPC* castration-resistant prostate cancer

profound downregulation of PTEN is needed for cancer progression to occur in the prostate [217]. These findings counter the canonical "two-hit hypothesis" of cancer and suggest that slight variations in PTEN expression, induced through genetic alterations as well as nongenetic changes in PTEN expression, are able to recapitulate varying stages of prostate tumor initiation and progression [218]. Despite the evidence for PTEN haplosufficiency in the mouse, evidence for this in humans still remains to be determined.

Phenotypes Associated with Homozygous Deletion of Pten in the Prostate Epithelium

A number of studies have been performed through the use of conditional mutants with prostate-specific deletion of one or both *Pten* alleles [211, 213–215, 219–221]. Conditional homozygous *Pten* deletion (*Pten*$^{-/-}$) driven by the *PB-Cre4* promoter

results in invasive adenocarcinoma in 100 % of mice at 9–12 weeks [215]. Importantly, the *Pten*$^{-/-}$ prostate cancer model mimics the course of human prostate cancer formation, progressing from hyperplasia to PIN to invasive adenocarcinoma with defined kinetics [215]. Interestingly, homozygous deletion of other tumor suppressors in the murine prostate, including *p53* [222], *retinoblastoma* (*Rb*) [223], and *Nkx3.1* [224], leads to PIN lesions but never an adenocarcinoma phenotype, solidifying the importance of PTEN function in the prostate gland. Moreover, although *Pten*-null tumors are initially responsive to androgen ablation, eventually mice will develop CRPC, as is commonly seen in human prostate cancer [215]. *Pten* homozygous deletion driven by other promoters in the mouse, including *PSA*^{Cre}, *MMTV*^{Cre}, and *Nkx3.1*^{CreERT2}, also results in the development of invasive adenocarcinoma, albeit over a longer latency [225–228].

Compound Pten Knockout Transgenic Mouse Models of Prostate Cancer

Pten Loss Combined with Alterations in Other Tumor Suppressors

Several studies carried out with compound transgenic mice have shown that monoallelic or biallelic deletion of tumor suppressor genes such as *Nkx3.1* [229, 230], *p27*^{KIP1} [231], and *p53* [105] can cooperate with *Pten* loss in promoting prostate cancer (Fig. 4.1). While loss of a single allele of *Nkx3.1* [224, 232] and *p27*^{KIP1} [233], both of which occurrences have been implicated in advanced stage prostate cancer and poor disease-free survival in humans [234, 235], is sufficient to promote prostate cancer initiation and PIN lesions, concomitant loss of *Pten* is needed to promote prostate tumorigenesis and cancer progression [211, 230, 231, 236]. Moreover, while the TRAMP mouse model alone, which contains inactivation of the *p53* and *Rb* tumor suppressor genes through expression of the large/small SV40 tumor T antigens under the *probasin* promoter, is capable of inducing the development of aggressive prostate tumors [209], loss of heterozygosity of *Pten* in TRAMP mice demonstrated an increased rate of tumor development, with a subsequent decrease in overall survival from 245 days to 159 days [237]. In the same way, conditional ablation of one or two alleles of *p53* leads to the development of PIN lesions, while *Pten*$^{-/-}$;*p53*$^{-/-}$ double mutants exhibit invasive prostate cancer as early as 2 weeks after puberty that is invariably lethal by 7 months of age [105, 238]. Also, deletion of *Smad4*, a tumor suppressor known to regulate the TGF-β signaling pathway, cooperates with *Pten* deletion in the prostate to enhance tumor cell proliferation and drive invasion to produce fully penetrant prostate cancer and metastases to the lymph nodes and lungs [239]. Finally, combining *Pten* and *p53* loss with loss of *Smad4* or reactivation of murine telomerase (*mTert*) produces prostate cancer metastases in the bone [240], indicating that additional pathway alterations are necessary to drive prostate tumor cells to form metastases in the microenvironment of the bone, an important feature of human prostate cancer.

Pten Loss Combined with Alterations in Oncogenes and Oncogenic Signaling Pathways

Activation of oncogenes and oncogenic signaling pathways cooperates with PTEN loss to promote invasive prostate cancer. In prostate cancer, the *ERG* gene is frequently translocated to the *TMPRSS2* promoter region, with the resulting *TMPRSS2–ERG* fusion protein expressed in 50 % of human prostate cancer specimens [241–243]. Whereas mice expressing *TMPRSS2-ERGa* under the control of the *ARR2Pb* promoter only develop PIN lesions [242], this translocation collaborates with *Pten* haplosufficiency to cause invasive adenocarcinoma of the prostate [244]. Similarly, cooperation between *FGF8b* overexpression and *Pten* haplosufficiency in a murine model leads to adenocarcinoma of the prostate, as well as lymph node metastases, in comparison to *FGF8b* overexpression alone, which leads to only hyperplastic and PIN lesions [245]. The 8q24 chromosomal region comprising the *MYC* oncogene is somatically amplified in a cohort of advanced human prostate tumors [246]. While mice engineered to express high levels of human *c-Myc* in the prostate (*PB-Cre4 Myc*^{hi}) develop invasive adenocarcinomas with 100 % penetrance [247], focal expression of *c-Myc* specifically in luminal epithelial cells of the prostate of mice (*PB-Cre4 Z-Myc*) results in only a mild pathology [248]. However, when combined with deletion of *Pten*, *PB-Cre4 Z-Myc* mice develop high-grade PIN and prostate cancer [248]. Although further investigation is needed to fully understand the synergistic effect of *c-Myc* activation and *Pten* loss in prostate cancer, evidence from this study and others suggests that loss of *Pten* may have differential effects depending on the cell types and regions/lobes/zones of the prostate where genetic deletion occurs. With the advent of cell type specific promoters in the prostate, future murine models will be able to tease out the effects of PTEN loss in specific cells in the prostate. For now, these models confirm that concomitant loss of *Pten* and genetic activation of oncogenes such as *ERG*, *FGF8b*, and *Myc* accelerate initiation and progression in human prostate cancer (Fig. 4.1).

Pten Loss Combined with Alterations in Inflammatory Pathway Regulators

Various lines of evidence suggest that chronic inflammation is linked to prostate tumorigenesis [249–251]. Indeed, expression of specific cytokines can be used as a prognostic indicator of biochemical recurrence in human prostate cancer [252]. One of the most prevalent inflammatory mediators clearly implicated in prostate cancer is IL-6, a cytokine that has not only been associated with tumor growth, proliferation, and angiogenesis in many cancers [253], but whose high levels in the circulating plasma of prostate cancer patients have also been correlated with advanced stages of the disease, therapeutic resistance, and an overall poor prognosis [254]. Although the foremost effect of IL-6 is activation of the JAK/STAT3 pathway [255], the PI3K/AKT pathway can also directly activate and phosphorylate STAT3 at Ser727 [256], which induces metastatic behavior of prostate cancer cells both in vitro and in vivo through stimulation of angiogenesis and suppression of

antitumor immune responses [257]. While transgenic mice that constitutively express *Stat3* under the control of the *ARR2Pb* promoter develop only PIN lesions, when crossed with *Pten*$^{+/-}$ mice, the subsequent compound mutants develop invasive prostate tumors [258].

Many inflammatory cytokines and chemokines promote tumor progression by converging on and stimulating the IKK2/NF-κB signaling axis [259]. The main function of IKK2 is the phosphorylation of IκB molecules, which act as inhibitors of NF-κB, thus rendering them subject to degradation and allowing NF-κB to remain activated. Constitutive activation of the transcription factor NF-κB in prostate cancer has been correlated with disease progression [260], and inhibition of NF-κB activity in prostate cancer cells can suppress angiogenesis and subsequent tumor invasion and metastasis by downregulating expression of downstream NF-κB targets such as VEGF and MMP9 [261]. Interestingly, while a mouse model containing a constitutively active version of IKK2 alone is insufficient in promoting prostate tumorigenesis, in combination with heterozygous loss of *Pten*, IKK2 activation leads to an increase in tumor size, accompanied by increased inflammation [262]. These studies demonstrate that inflammatory cytokines secreted from the stromal microenvironment of the prostate cooperate with PTEN loss to drive epithelial prostate tumor cells toward invasive disease.

PTEN and Tumor Cell Migration and Invasion

As demonstrated in various models of conditional *Pten* deletion in the prostate, homozygous *Pten* loss leads to progression from PIN lesions to invasive adenocarcinoma, a process that requires disruption of the basement membrane and junctional integrity in epithelial acinar structures to allow the invasion of tumor cells through the surrounding basement membrane and into the stromal microenvironment (Fig. 4.1). PTEN and PIP3 play conserved roles in the determination of cell polarity in diverse cell types. From data first obtained in *Dictyostelium discoideum* [263–266], a unicellular amoeba, and later from neutrophils undergoing chemotaxis [267, 268], enrichment of PIP3 at the leading edge of migrating cells and localization of PTEN in the lateral and trailing edges of the cell has been observed. The PI3K pathway also promotes membrane ruffling, cell motility, and cellular spreading through downstream effectors such as RHO, RAC1, and CDC42 [269]. Consequently, forced expression of PTEN in tumor cell lines inhibits tumor cell invasiveness in vitro and in xenografts in vivo through both phosphatase-dependent [110, 270] and phosphatase-independent [102] mechanisms. In normal glandular development, PTEN concentrates to the apical plasma membrane during epithelial morphogenesis, where it catalyzes the conversion PIP3 into PIP2, which recruits ANX2, CDC42, and aPKC to the membrane to establish cellular polarity [271]. In this regard, loss of PTEN expression may block the development of the apical surface and lumen of epithelial structures. Therefore, activation of the PI3K/AKT pathway upon PTEN loss may lead to the loss of epithelial features, and thereby increase the likelihood

of cells developing the properties of increased motility and invasive capacity through an epithelial-to-mesenchymal transition (EMT) [128]. In all, these findings raise the possibility that the considerable increase in the *PTEN* mutation/deletion rate in metastatic tumors might result from a selective metastatic advantage acquired through the loss of PTEN regulation of motility and invasion.

Pten Loss in Metastatic Prostate Cancer Mouse Models

It is clear from these models that *Pten* LOH is required for cancer progression and invasive adenocarcinoma development. Although biallelic *Pten* deletion, alone or in combination with homozygous deletion of *p53* [105, 238], *Nkx3.1* [230], *or Smad4* [239] or activation of *FGF8b* [245], does lead to the occurrence of small micrometastases in the lymph nodes and lungs, it fails to produce significant metastatic burden, particularly in the bone [215]. Therefore, other genetic alterations and signaling pathway abnormalities must collaborate with activation of the PI3K/AKT pathway to promote metastatic prostate cancer to the bone.

Although *Ras* mutations [272–274] and *Ras* fusion events [275] in prostate cancer are uncommon, strong evidence suggests that Ras/MAPK activation plays a substantial role in human prostate cancer progression, particularly in metastasis and CRPC development. Indeed, the Ras/Raf/MAPK pathway has been recently shown to be altered in 43 and 90 % of primary and metastatic lesions, respectively [26]. P-MAPK levels, as assessed in tumor microarrays (TMAs) from human prostate cancer samples, are significantly elevated in neo-adjuvant treated, recurrent, and CRPC patients as compared to benign prostatic hyperplasia (BPH) specimens, corresponding with a significant reduction in PTEN expression [122]. These findings have prompted the development of two recent murine models of prostate cancer that combine homozygous *Pten* loss with activation of the Ras/Raf/MAPK pathway: the *PbCre;Pten*$^{L/L}$*;Kras*$^{G12D/+}$ model [122], and the *Nkx3.1*$^{CE2/+}$*;Pten*$^{f/f}$*;Braf*$^{CA/+}$ model [276]. In both models, activation of the MAPK pathway through either *Braf* or *Kras* conditional overexpression resulted in overt macrometastases to the lymph nodes, lungs, liver, and, importantly, the bone marrow, in around 30 % [276] and 100 % [122] of cases, respectively. In the *PbCre;Pten*$^{L/L}$*;Kras*$^{G12D/+}$ model, treatment with a MEK inhibitor alone was able to fully ablate metastatic spread to the lungs and other distant organs, implicating the RAS/RAF/MAPK pathway as a driver of metastasis in *Pten*-deficient prostate cancer [122]. Interestingly, an EMT phenotype is also observed at the primary tumor site in the *PbCre;Pten*$^{L/L}$*;Kras*$^{G12D/+}$ model [122]. As EMT has been postulated to play a critical role in the process of metastasis [277], this new model provides a unique opportunity to study the impact of EMT in prostate cancer metastasis in vivo in the context of *Pten* loss and Ras/MAPK activation. With these novel metastatic models of prostate cancer, a better understanding of the contribution of PTEN to the metastatic cascade, including localized invasion, intravasation into the blood stream, survival as circulating tumor cells (CTCs), extravasation out of the blood stream, and metastatic seeding to distant

organ sites, can be further uncovered. Overall, past and present murine models of prostate cancer induced by *Pten* loss have demonstrated that loss of PTEN, to varying degrees and in combination with other genetic alterations, can recapitulate the entire spectrum of prostate cancer, from initiation (heterozygous *Pten* loss), through progression (homozygous *Pten* loss), and, finally, to metastasis (homozygous *Pten* loss and Ras/MAPK activation) (Fig. 4.1).

PTEN and CRPC

Androgens are indispensable for prostatic glandular development and homeostasis and contribute to prostate cancer development through activation of the androgen receptor (AR). Androgen deprivation therapy remains the most common treatment for advanced prostate cancer. However, therapeutic effects are short lived, and patients usually succumb to CRPC within 18–24 months, leaving the disease essentially untreatable [278]. New generation androgen deprivation therapies (ADTs), such as abiraterone [279] and MDV3100 [280], that more effectively ablate androgen production and AR signaling, are rapidly being developed and approved for patients with metastatic CRPC. Similar to human prostate cancers, while castration initially results in massive apoptosis of the prostate epithelium in the *Pten*-null murine model of prostate cancer, the ki67 proliferation index remains constant, indicating that a select population of cells remains resistant to androgen withdrawal [215].

AR is expressed in CRPC and may function through autocrine signaling or crosstalk with other prosurvival and proliferation pathways [281, 282], including the PI3K/AKT pathway, which has been shown to induce AR expression in the absence of PTEN [283–285]. Multiple studies have found an association between the loss of PTEN and the development of CRPC [201, 215, 286, 287]. Moreover, loss of PTEN and AR expression has been correlated clinically with increased mortality in CRPC patients [193]. aCGH analysis on metastatic prostate cancer samples has also demonstrated frequent amplification of AR (73 %), coinciding with aberrant deletion of PTEN (87 %) [288].

While some studies have proposed that PTEN deletion activates AR through PI3K-mediated stabilization of AR protein levels or AKT-mediated phosphorylation and activation of the AR [231, 289, 290], other reports have revealed that PI3K/AKT pathway stimulation promotes degradation of AR and inhibits AR transcriptional activity [291]. Supporting the later claim, levels of AR are heterogeneous, and, in many cases, absent in late stage, metastatic disease [292–295]. These observations raise the possibility that loss of AR expression and activity may serve as a means of evading androgen withdrawal through simultaneous activation of other signaling pathways. Indeed, two independent laboratories have recently demonstrated that PTEN loss inhibits androgen-responsive gene expression by regulating AR activity [296, 297], indicating that castration-resistant growth is an intrinsic property of *Pten* null prostate cancer cells regardless of cancer stage [296]. These studies further suggest a reciprocal feedback loop that exists between AR and PTEN

in prostate cancer, in which conditional deletion of *Ar* in the prostate epithelium promotes the proliferation of *Pten*-deficient cancer cells in *PbCre;Pten*$^{L/L}$*;Ar*$^{L/Y}$ mice through the downregulation of the androgen-responsive gene *Fkbp5* and preventing PHLPP-mediated AKT inhibition [296]. Moreover, inhibition of the PI3K/AKT pathway was shown to upregulate the receptor tyrosine kinase HER3 [297]. As suppression of HER2/HER3 heterodimers has been linked to inhibition of AR transcriptional activity through an AKT-independent mechanism [298], it is plausible that PI3K/AKT inhibition upregulates AR transcriptional activity by increasing HER3 expression.

In all, it is probable that AR suppresses the PI3K/AKT pathway in order to promote differentiation of the prostate epithelium and keep prostate cancer cells sensitive to androgens. When AR activity is downregulated upon ADT treatment, the PI3K/AKT pathway takes over to promote cell proliferation and cell survival in the absence of androgen or AR activity, further driving tumor progression toward metastatic CRPC [296, 297]. These findings may explain why clinical trials that inhibit the activation of the PI3K/AKT signaling axis, as well as its downstream effector mTOR, failed to have a substantial effect on tumor progression in men [299, 300], as inhibition of the PI3K/AKT/mTOR pathway causes an upregulation of AR transcriptional activity that promotes cell survival [297]. Since, in the background of PTEN-deficient prostate cancer, AKT regulates proliferation, while AR regulates survival, inhibition of both signaling pathways is necessary for effective tumor reduction. Indeed, combined therapy targeting both PI3K and AR pathways reduces tumor growth in *Pten*-null mice [296, 297], suggesting the possible efficacy of combined PI3K/AKT and AR inhibitor treatment in the clinic (Fig. 4.1).

PI3K/AKT/mTOR Pathway Inhibition as a Treatment for Prostate Cancer

Current Prostate Cancer Treatment

Treatment resistance is a major issue in the management of prostate cancer, as it is estimated that 30,000 men in the USA will die in 2012 alone from metastatic and CRPC, for which there is currently no cure. Although androgen-deprivation therapy (ADT) remains the standard treatment of metastatic prostate cancer, progression to castration-resistant disease occurs in the majority of patients [301–303]. Among available therapeutic approaches for the treatment of CRPC, conventional chemotherapy with docetaxel and other agents has limited efficacy and has yet to produce long-term benefits [304, 305]. Although agents that specifically inhibit the AR, androgen synthesis, and/or AR-regulated pathways, such as MDV3100 and Abiraterone, have recently entered the clinic and have shown promising results [279, 280], their therapeutic effects are short lived, and patients eventually develop CRPC [278]. Another novel therapy, sipuleucel-T, which is the first ever FDA-approved therapeutic cancer vaccine for the treatment of metastatic prostate

cancer, also only modestly improves the survival of late-stage patients by a few months [306, 307].

The current trend in medicine has been to exercise a personalized treatment approach that is based on molecular and genetic profiling of individual patients to determine the best therapeutic strategy. A considerable number of novel therapeutics are presently undergoing clinical trials, including small molecules that target common genetic or pathway alterations found in human cancers. These inhibitors have been FDA approved for treatment of various solid tumors including, renal, GIST, breast, pancreatic, colorectal, and NSCLC cancer [308–314], and thus hold promise for the treatment of prostate cancer. As it is clear that PI3K/AKT/mTOR pathway activation plays a prominent role in prostate cancer initiation and progression, CRPC, and metastatic disease, the loss of PTEN expression in individuals with prostate cancer could be used as a biomarker to stratify populations of patients that may benefit from treatment with PI3K/AKT/mTOR pathway inhibitors.

PI3K Inhibitors

Class I PI3Ks are heterodimers composed of one catalytic subunit of p110α, p110β, or p110δ, collectively known as p110, and a regulatory subunit, p85. PI3K isoform selectivity may be essential to boost therapeutic efficacy and minimize off-target toxicity. Recent research suggests a dominant role for the PI3K isoform p110β in PTEN-deficient tumors. In the *Pten*-null prostate cancer model, loss of p110β, but not p110α, decreased PI3K signaling and prevented prostate carcinogenesis [94]. In a similar fashion, inducible depletion of p110β, but not p110α, using shRNA in PTEN-deficient human cancer cell lines quenches PI3K-mediated signaling and inhibits growth both in vitro and in vivo [315].

The most studied PI3K inhibitors to date are the first-generation PI3K inhibitors LY294002 and wortmannin. LY294002 treatment results in cell-cycle arrest and sensitizes the LNCaP prostate cancer cell line to radiation therapy, decreases the invasive properties of LNCaP, PC-3, and DU145 prostate cancer cell lines, and inhibits angiogenesis in PC-3 prostate cancer cells by way of decreased levels of HIF1-α and VEGF. Similarly, wortmannin induces apoptosis and radiosensitizes DU145 cells [316, 317]. However, both wortmannin and LY294004 show limited selectivity for individual PI3K isoforms, nonspecifically target multiple other signaling molecules [318–321], and demonstrate significant toxicity in animals [317, 322], limiting their effectiveness in vivo.

One potential consequence and side effect of PI3K pathway inhibition is the development of insulin resistance in patients. While both p110α and p110β play specific roles in insulin signaling, research suggests that glucose homeostasis is predominantly mediated by p110α [94, 323]. Indeed, p110α inhibitors, but not p110β or p110δ inhibitors, alter insulin-dependent glucose regulation in mice [323]. Thus, in the setting of PTEN-deficient tumors, p110β-specific inhibitors, in contrast to pan-PI3K inhibitors, may offer enhanced efficacy with a reduced likelihood of insulin resistance. Together, these studies suggest that effective treatment of

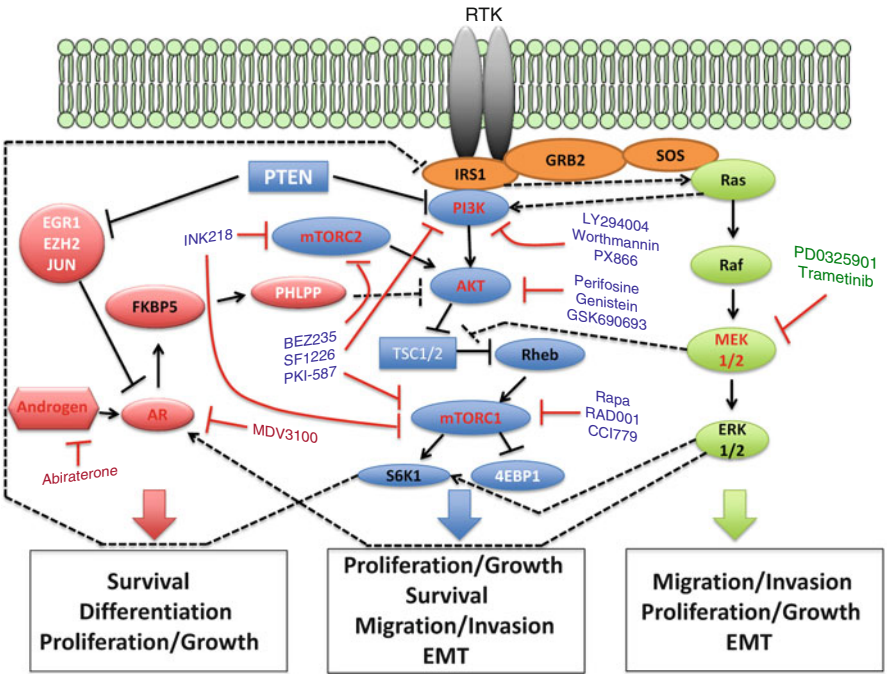

Fig. 4.2 *PI3K/AKT/mTOR, Ras/MAPK, and AR signaling pathways converge to promote prostate cancer development.* Although all three pathways promote cell proliferation/growth, AR signaling maintains prostate cells in a differentiated state, while PI3K/AKT/mTOR and Ras/MAPK signaling promotes EMT and cell migration/invasion. *Red*, *blue*, and *green ovals* represent AR, PI3K/AKT/mTOR, and Ras/MAPK signaling molecules, respectively. *Orange ovals* denote adaptor molecules. Pathway activators are in *black letters*, and pathway suppressors are in *white letters*. *Solid black lines* depict signaling within a pathway, and *dotted black lines* depict crosstalk between pathways or feedback loops. *Red lines* denote the drug targets. Signaling molecules in these pathways that are the targets of drug inhibitors are in *red letters*

PTEN-deficient prostate tumors may necessitate the use of therapeutic agents that successfully target p110β. However, even in cancers that may be specifically reliant on either p110α or p110β, there remains the possibility that other, noninhibited p110 isoforms may make up for the decreased activity of the targeted isoform. Moreover, not all tumors that are driven by PTEN loss are dependent on p110β, and the presence of other genetic modifications and pathway alterations is likely to change the PI3K-isoform reliance of these tumors. Interestingly, PTEN loss appears to be a predictive marker for sensitivity to PX-866, an oral derivative of wortmannin, despite the fact that PX-866 displays a high efficacy against p110α and p110δ but not p110β [324]. Therefore, although PI3K signaling is an obvious target for therapy, especially in PTEN-deficient prostate cancer, given the redundancy and complex feedback regulation existing in the PI3K/AKT/mTOR pathway, as well as a need for a more in-depth understanding of the pathway, the clinical efficacy of using PI3K inhibitors as single agents is modest (Fig. 4.2).

AKT Inhibitors

The significance of the individual AKT isoforms in prostate cancer has yet to be fully uncovered, despite findings that AKT-1 isoform expression may be a prognostic marker for biochemical recurrence in patients with prostate cancer [325]. There are several classes of AKT inhibitors currently in development, including isoform-selective AKT catalytic–domain inhibitors and inhibitors of its PH domain, and many have been tested in prostate cancer. Perifosine, an alkylphospholipid that targets the PH domain of AKT and prevents it from binding to PIP3, decreases AKT phosphorylation, inhibits growth, and induces cell-cycle arrest of PC-3 cells [326]. Although there are no published preclinical studies investigating perifosine activity against prostate cancer, perifosine has gone to clinical trials for patients with CRPC. Though generally well tolerated, perifosine showed no evidence of significant inhibitory activity [300, 327]. Genistein, a non-specific AKT inhibitor, causes significant growth inhibition and apoptosis of cancer cells [328, 329]. While genistein has demonstrated significant potential in vivo, decreasing the incidence of lung metastasis in an orthotopic model using PC-3 cells [330] and inhibiting tumor growth when combined with docetaxel in an experimental model of bone metastasis [331], another report claimed that genistein increased the size of metastatic lymph nodes in a PC-3 orthotopic model [326]. Concomitant targeting of AKT-1 and AKT-2 with ATP-competitive inhibitors, such as GSK690693, has been shown to be more effective than inhibition of single isoforms for the induction of apoptosis in tumor cells, suggesting that these Pan-AKT inhibitors are likely to have more promise in the clinic, although increased toxicity may be a potential issue [332]. However, AKT inhibitors will not block the non-AKT effectors downstream of PI3K signaling. Paradoxically, AKT inhibitors could increase upstream receptor tyrosine kinase activities by alleviating downstream negative feedback loops [333]. Therefore, the importance of AKT-independent effectors of PI3K signaling and downstream negative feedback loops in the pathway might considerably affect the clinical effectiveness of AKT inhibitors (Fig. 4.2).

mTOR Inhibitors

mTOR inhibitors have been the most effective among the inhibitors of the PI3K/AKT/mTOR pathway in treating solid tumors and have received the most consideration in the treatment of prostate cancer. Rapamycin, the prototypical mTOR inhibitor, associates with its intracellular receptor, FKBP12, which then binds directly to mTORC1 and suppresses mTOR-mediated phosphorylation of its downstream effectors, S6K and 4EBP1. Rapamycin induces cell-cycle arrest in PC-3 and DU145 prostate cancer cell lines in vitro [334–337] and reduces tumor volume and blocks growth and proliferation in tumors with activated AKT or loss of PTEN in vivo [338, 339]. Although limited, there have been reports on in vitro and preclinical

studies demonstrating the efficacy of the rapamycin analogs (rapalogs) CCI-779 and RAD001 in the treatment of prostate cancer. CCI-779 inhibits growth of PC-3 and DU145 cells in vitro, and, in vivo, and reduces tumor volumes in PC-3 and DU145 xenografts [340]. Likewise, RAD001 treatment decreases proliferation of prostate cancer cells in vitro [341, 342] and reverses PIN lesions in AKT-1 transgenic mice [343].

Despite these preclinical findings, mTORC1 inhibitors, including rapamycin and rapalogs, have demonstrated little success as single agent treatments in the clinic [299, 344, 345]. Although rapamycin and rapalogs are effective at inhibiting mTORC1 kinase activity, inhibition of mTORC1 eventually leads to AKT activation and increased P-AKT levels due to the loss of the S6K to IRS-1 feedback loop and reactivation of PI3K signaling [41, 345, 346]. Moreover, while mTORC1 is sensitive to rapamycin treatment, mTORC2 is generally considered to be resistant to rapamycin. In this regard, mTORC2 phosphorylation and activation of AKT may further limit the efficacy of mTORC1 inhibitors like rapamycin [38]. Therefore, the use of rapamycin and rapalogues as single treatments could potentially cause the hyperactivation of the PI3K/AKT pathway (Fig. 4.2).

To achieve a significant clinical effect, mTORC1 inhibition with rapamycin and rapalogs may require the addition of upstream inhibitors, such as insulin-like growth factor signaling or PI3K signaling inhibitors [347–350], or, alternatively, more effective inhibition of both TORC1 and TORC2 activity. mTOR catalytic site inhibitors, which are currently in clinical development, target the kinase domain of mTOR and have the advantage of blocking the activity of both mTORC1 and mTORC2. The additional inhibition of mTORC2 provides the benefit of blocking AKT activation through S473 phosphorylation, and therefore, these catalytic site inhibitors would be expected to inhibit the mTOR pathway more effectively than rapamycin. Current research has described torkinibs and torin1, two selective ATP-competitive inhibitors of mTOR that impede cellular growth and proliferation more effectively than rapamycin [351, 352]. Interestingly, however, the enhanced activity of these mTOR kinase inhibitors seems to be due to more complete inhibition of mTORC1 activity rather than mTORC2 inhibition, as measured by decreased levels of 4EBP1 phosphorylation and cap-dependent translation compared to rapamycin treatment [351, 352]. In support of the efficacy of these mTOR catalytic site inhibitors, a recent preclinical study using the mTOR catalytic site inhibitor INK218 in the *Pten*-null murine prostate cancer model demonstrated that INK218 is able to inhibit AKT and 4E-BP1 phosphorylation in addition to S6K1 phosphorylation, lead to a 50 % decrease in PIN lesions, reduce overall tumor volume, and promote tumor cell apoptosis, as opposed to RAD0001 treatment, which results in inhibition of S6K1 but not AKT and 4E-BP1 phosphorylation, only partial regression of PIN lesions, and no significant effect on tumor cell apoptosis [353]. Remarkably, treatment with INK218 blocks progression of invasive prostate cancer locally in the prostate and even inhibits the total number and size of distant metastases [353]. Although new generation mTOR catalytic site inhibitors have the capacity to reduce prostate tumor invasion and metastasis by more effectively disabling mTORC1 signaling and inhibiting mTORC2 activation, treatment with these inhibitors alone

does not inhibit PI3K activity, and, therefore, would need to be combined with other PI3K antagonists to fully ablate distant metastasis and lead to complete tumor regression (Fig. 4.2).

Dual PI3K–mTOR Inhibitors

The use of multiple inhibitors to target the PI3K/AKT/mTOR pathway may be of particular importance to alleviate the issue of negative-feedback loops in the pathway. As the catalytic domains of the p110 subunits and mTOR are similar in structure, there are a number of small molecule inhibitors currently being tested that can block both PI3K and mTOR. Compared to other PI3K pathway inhibitors, dual PI3K–mTOR inhibitors, which include NVP-BEZ235, BGT-226, XL765, SF1126, PKI-402, and PKI587, have the possible advantage of inhibiting all PI3K isoforms, as well as both mTORC1 and mTORC2. Therefore, these inhibitors should effectively turn off the PI3K/AKT/mTOR pathway completely and overcome feedback inhibition normally observed with mTORC1 inhibitors such as rapamycin and other rapalogs [347]. BEZ235 is capable of simultaneously inhibiting multiple class I PI3K isoforms and mTOR kinase activity by binding to their respective catalytic sites [349]. BEZ235, unlike PI3K inhibitors alone, is able to lower levels of both P-S6 and P-AKT, demonstrating that dual inhibition of both mTOR and PI3K is capable of preventing an increase in P-AKT levels [349, 350]. BEZ235 exhibits greater antiproliferative effects compared with rapamycin treatment in cancer cell lines in vitro and slows tumor growth and vasculature development in PTEN-deficient cell line engrafted mice, where it is well tolerated with no significant changes in body weight [349, 350, 354]. In preclinical studies, SF1126, a conjugate of LY294002, reduces cell growth, proliferation, and angiogenesis, and exhibits lower toxicity than LY294002 [355]. Furthermore, PKI-587, another dual PI3K/mTOR inhibitor, induces tumor regression in several cancer cell line xenograft models, and has a favorable drug safety profile in toxicology studies [356]. Importantly, in contrast to PI3K inhibitors that cause cytostatic effects through tumor cell G_0–G_1 arrest [357–359], PKI-587 inhibition of PI3K and mTOR can fully ablate AKT activation and cause the induction of apoptosis, the preferred outcome against tumor cells [356]. Despite these preclinical findings, a major issue with dual PI3K/mTOR inhibitors is their efficacies in vivo in the clinical settings.

Combination Therapy with PI3K/AKT/mTOR and Ras/MAPK Inhibitors

One explanation behind the limited success of PI3K/AKT/mTOR pathway inhibition in the clinic is that blockade of PI3K signaling may shift the tumor survival signaling to a Ras/MAPK-dependent pathway [360]. Analyzes of human prostate cancer

microarrays have demonstrated that the PI3K/AKT and Ras/MAPK pathways are often coordinately dysregulated during prostate cancer progression in humans [122, 361]. Although BEZ235 is effective against PI3K-driven tumors as a single agent, the inhibitor responds poorly to tumors harboring Kras mutations [350]. Indeed, BEZ235 is only effective against Kras-driven tumors when combined with a MEK inhibitor [362]. Humans with advanced prostate cancer treated with RAD001 experience increased activation of MAPK signaling, probably due to the loss of the S6K-IRS1 feedback loop that leads to Ras activation [360] (Fig. 4.2). In addition, neoadjuvant hormone therapy can lead to increased P-MAPK activation and N-cadherin expression, both of which have been implicated in the induction of the EMT program and metastatic prostate cancer [122, 363]. Ras activation can also play a direct role in moving prostate cancer cell lines toward decreased androgen dependence [364]. Indeed, PI3K/AKT and Braf/ERK pathway activation acts combinatorially in a mouse model of CRPC [365]. These studies suggest the importance for combined PI3K/AKT and Ras/MAPK pathway blockade in the treatment of CRPC and metastatic prostate cancer.

A number of studies conducted with *Pten* knockout mice have shown that combined pharmacological targeting of mTOR and MEK may lead to reduced primary prostate tumor progression [122, 361]. Combination therapy using the mTORC1 inhibitor rapamycin and the MEK inhibitor PD0325901 inhibits not only growth in prostate cancer cell lines [361] but also reduces tumor burden in castrated, androgen-insensitive prostate tumors in the $Nkx3.1^{-/-};Pten^{+/-}$ murine model [365]. Dual mTOR and MEK inhibition also completely ablates the dissemination of distant metastases in the $PbCre;Pten^{L/L};Kras^{G12D/+}$ murine prostate cancer model, which exhibits 100 % penetrable macrometastasis [122], as well as reduces tumor and metastatic burden in $Nkx3.1^{Cre\text{-}ER};Pten^{f/f};Braf^{CA/+}$ mice [276]. Thus, in late stage, metastatic prostate cancer, dual PI3K/AKT and Ras/MAPK inhibition may be necessary to reduce metastasis, as well as slow primary tumor growth (Fig. 4.1).

Combination Therapy with PI3K/AKT/mTOR and AR Pathway Inhibitors

Recent studies using the *Pten*-null murine model of prostate cancer have demonstrated a reciprocal feedback loop that exists between AR and PI3K pathways in the prostate cancer, whereby inhibition of the PI3K pathway in *Pten*-deficient prostate cancer results in reactivation of AR signaling by modulating AR corepressor activities or through feedback signaling to the receptor tyrosine kinase HER2/HER3 [296, 297]. Therefore, the efficacy of PI3K inhibitors for the treatment of *PTEN-deficient* prostate cancer may be improved through combined AR pathway inhibition. Another recent study utilizing surgical castration in *Pten*-null mice to model CRPC demonstrated that dual targeting of both AKT and mTOR with inhibitors MK-2206 and MK-8869, respectively, is highly effective at inhibiting CRPC in vivo [366]. Moreover, the AR agonist MDV3100, which has shown promise in the clinic,

has improved efficacy in combination with BEZ235 [350], a dual inhibitor of PI3K and mTORC1/2, in castration-resistant GEM mice [297]. Other laboratories have also documented beneficial effects of combined AR and mTORC1 inhibition with rapamycin in $Pten^{-/-}$ models [296, 367]. Thus, more effective inhibition of the AR signaling axis with new generation inhibitors such as abiraterone and MDV3100 in combination with mTOR or PI3K/mTOR dual inhibitors may prove to be more beneficial in treating CRPC patients displaying alterations in PI3K/AKT pathway signaling (Fig. 4.2).

In all, although dual PI3K/mTOR inhibitors now offer the advantage of complete PI3K/AKT/mTOR pathway inhibition, with signaling feedback loops present in the PI3K/AKT/mTOR pathway that negatively control both Ras/MAPK and AR signaling, it is likely that PI3K inhibition alone will not be able to achieve full regression of tumors in patients with prostate tumors driven by PTEN loss. A better understanding of pathway dynamics gained from recent preclinical studies prompts the rationale for combining inhibition of the PI3K/AKT/mTOR pathway with inhibition of either the Ras/MAPK or AR signaling pathways for the treatment of metastatic CRPC. However, better surrogate biomarkers that predict patient responses to PI3K inhibitors, as well as more high-throughput systems to molecularly profile and detect PTEN loss or PI3K/AKT/mTOR activation in patients, will be needed to accurately assess the efficacy of PI3K/AKT/mTOR inhibition as a treatment in individual patients.

Conclusions and Perspectives

In the 15 years since the discovery of PTEN as a frequently mutated gene in cancer, great progress in understanding the function and regulation of PTEN has been made. While PTEN was first identified as a lipid phosphatase with tumor suppressive activity against the PI3K/AKT pathway, recent studies have revealed that PTEN has additional protein phosphatase and lipid phosphatase-independent activities, as well as functions in the nucleus. Further understanding of the mechanisms behind PTEN post-transcriptional regulation, post-translational modifications, and protein–protein interactions offers novel therapeutic opportunities, as well as explanations of why, clinically, loss of PTEN expression can occur without genetic deletion or mutations at the *PTEN* locus. With improved methods for detecting PTEN status, such as CGH, whole-exome sequencing, and transcriptome analysis, PTEN loss can more readily and more accurately be used as a prognostic biomarker for men with prostate cancer, as well as a potential predictive marker to identify patients who could benefit from emerging PTEN/PI3K/Akt pathway therapies. Moreover, studies with large human prostate cancer cohorts have revealed that alterations in the PI3K/AKT/mTOR pathway are more common in advanced, metastatic prostate cancer and CRPC compared to primary, androgen-dependent tumors and are associated with a poorer overall prognosis and increased chance of biochemical recurrence. Recent works using mouse models with varying degrees of *Pten* loss have helped to

reveal the role of PTEN dosage in prostate tumorigenesis and the collaborative effects of PTEN loss and other genetic and pathway alterations in prostate cancer initiation, progression, and metastasis. A better understanding of the interactions between the PI3K/AKT pathway and Ras/MAPK, p53, and TGF-β–Smad pathways has facilitated the development of metastatic models of prostate cancer with bone metastasis potential, an important feature of human prostate cancer. As bone tropism of prostate cancer metastasis is not well understood, these mouse models should provide better insights into the cell types and molecular pathways involved in metastasis to the bone. Better systems, via lineage-tracing and cell type-specific deletion, are needed to address which cell types are responsible for different stages of the disease, including prostate cancer progression, castration resistance, and metastasis. As previous studies have suggested that prostate luminal, transit amplifying (TA), and basal cells can serve as a cell-of-origin and as cancer stem cell (CSC) populations in prostate cancer [368], it will be important for future models to employ more restrictive prostate-specific promoters allowing the potential for tumor initiation from basal, TA, neuroendocrine, and luminal cell types. Two recent reports have also elucidated a reciprocal feedback loop between the PI3K/AKT and AR signaling pathways that directly regulates CRPC, offering an explanation for how loss of androgen dependence may further strengthen PI3K/AKT signaling in PTEN-deficient prostate cancer, as well as a rationale for the combined use of AR signaling and PI3K/AKT/mTOR inhibitors in the treatment of CRPC.

Although beyond the scope of this review, emerging research in other solid tumors has demonstrated that the tumor microenvironment itself may play a defined role in tumor propagation and progression, and it will be interesting to see if aberrations in the PI3K/AKT/mTOR signaling in stromal-specific subtypes themselves in the prostate may contribute to tumorigenesis. Moreover, PTEN alterations in the tumor epithelium, which have been demonstrated to induce the release of paracrine signals, including chemokines and cytokines that may attract immune cell types to the prostate and contribute to the development of a tumor-permissive rather than a tumor-suppressive microenvironment, suggest that immunotherapy may be a possible treatment for prostate cancer patients. Again, the specific stromal cells and immune cells that contribute to the prostate tumor microenvironment will need to be further pursued with the use of lineage-specific promoters and tracking systems in immune competent models that preserve the tumor's native microenvironment.

Finally, while past clinical trials using rapamycin and rapalogues to treat human prostate cancer have shown little efficacy, due in part to an inability to inhibit PI3K and AKT signaling, dual PI3K/mTOR inhibitors have the capacity to completely inhibit all strands of the PI3K/AKT/mTOR pathway, and thus deserve further study in preclinical models of prostate cancer. However, with signaling feedback loops present in the PI3K/AKT/mTOR pathway that negatively control both Ras/MAPK and AR signaling, it is unlikely that PI3K inhibition alone will be able to achieve full regression of PTEN-deficient prostate tumors. Further understanding of pathway dynamics gained from recent preclinical studies prompts the rationale for combining inhibition of the PI3K/AKT/mTOR pathway with inhibition of either the Ras/MAPK or AR signaling pathways for the treatment of metastatic CRPC. In the

end, though much progress has been made in understanding the role PTEN and its regulation of the PI3KAKT/mTOR pathway in prostate cancer, in the future, more basic and preclinical mechanistic studies that further elucidate the complexity of the PI3K/AKT/mTOR signaling pathway and can be translated from bench to bedside will help to design better treatment options for patients with metastatic CRPC, for which there is still no cure.

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Chapter 5
ETS Fusion Genes in Prostate Cancer

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Abstract In 2005, we reported the discovery of recurrent chromosomal rearrangements resulting in the fusion of the 5′ untranslated region of an androgen regulated gene, *TMPRSS2*, with members of the ETS transcription factor family (*ERG* or *ETV1*). After nearly a decade, enormous progress has been made in understanding the diversity of ETS gene fusions, their genesis, and oncogenic roles in vitro and in vivo. Similarly, ETS gene fusions provide a rational basis for the comprehensive molecular subtyping of prostate cancer, especially in light of recently described co-occurring or mutually exclusive genetic events, which may have utility in risk prediction as well as therapeutic targeting. Given their remarkable cancer specificity, ETS gene fusions have enormous potential as biomarkers, and clinical translation is ongoing. We discuss the utility of ETS gene fusions for both tissue based diagnosis, risk stratification of precursor lesions, and early detection. Similarly, given the attention on distinguishing "aggressive" from indolent prostate cancer, we have reviewed the potential of ETS gene fusions in this context and focus on the important caveats required to interpret such studies, including the ETS gene fusion detection method, the clinical cohort characteristics, and how aggressiveness and outcome are determined. Importantly, these factors are important not just for evaluating ETS fusions, but all prostate cancer biomarkers. Lastly, as the most prostate cancer-specific biomarker yet reported, we review the potential of ETS gene fusions as both indirect and direct therapeutic targets.

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Introduction

In 2005, we reported the discovery of recurrent chromosomal rearrangements resulting in fusion of the androgen-regulated gene *TMPRSS2*, and members of the ETS family members (most commonly *ERG* and *ETV1*), in almost half of all prostate cancers [1]. Although well described in hematological and soft tissue malignancies, this was the first report of recurrent chromosomal rearrangement in common epithelial carcinomas (i.e., breast, colon, lung, and prostate) that cause the majority of cancer morbidity and mortality in the USA This discovery has not only transformed our understanding of prostate cancer biology but is enabling clinical advances in the molecular subtyping of prostate cancer, prostate cancer detection and diagnosis, and clinical management. Several outstanding reviews have been published on ETS gene fusions in prostate cancer [2–12], so here we will focus on reviewing topics of broad interest and those not comprehensively assessed in prior reviews.

Discovery of *TMPRSS2-ETS* Gene Fusions in Prostate Cancer

By the mid 2000s, global gene expression profiling by DNA microarrays was a standard method to characterize the cancer transcriptome and was utilized to classify histologically similar tumors [13–16], identify cancer-specific biomarkers [17], and predict the behavior of tumors [18–20]. However, relatively few causal cancer genes had been discovered using DNA microarrays. In 2005, we hypothesized that causal cancer genes activated by amplification (such as *ERBB2*) or rearrangements (such as *BCR:ABL1*) should be identifiable in cancer gene microarray data due to relative overexpression in cases that harbor these events. However, standard analysis of cancer gene expression profiling data was predominantly designed to answer questions such as which gene is most consistently overexpressed in all cancers compared to normal samples, or which gene is most consistently overexpressed in all cancers with poor outcome compared to cancers with good outcome. Yet, the majority of causal cancer genes are not activated in all instances of a given cancer type—for example, *ERBB2* is amplified in only a fraction of breast cancers, *BRAF* is only mutated in approximately half of all melanomas, and even the *BCR:ABL1* fusion is present in only a subset of all leukemias (which can be subdivided from other leukemias based on morphology and complementary approaches).

Thus, we developed a method, termed cancer outlier profile analysis (COPA), to identify genes that showed the expression pattern characteristic of causal cancer genes: very high expression in a *fraction* of cancer samples. Importantly, COPA utilizes normalization based on median absolute deviation of gene expression to accentuate outlier profiles (reviewed in [5]). We then applied this method to the Oncomine database of cancer microarray studies (http://www.oncomine.com) and prioritized known cancer causing genes showing high ranking outlier profiles in any cancer profiling study. In addition to prioritizing outliers correctly associated with cancer types, such as *ERBB2* in breast cancer and *RUNX1T1* (also known as *ETO*) in acute myeloid leukemia (only in samples harboring *AML1:ETO* chromosomal rearrangements),

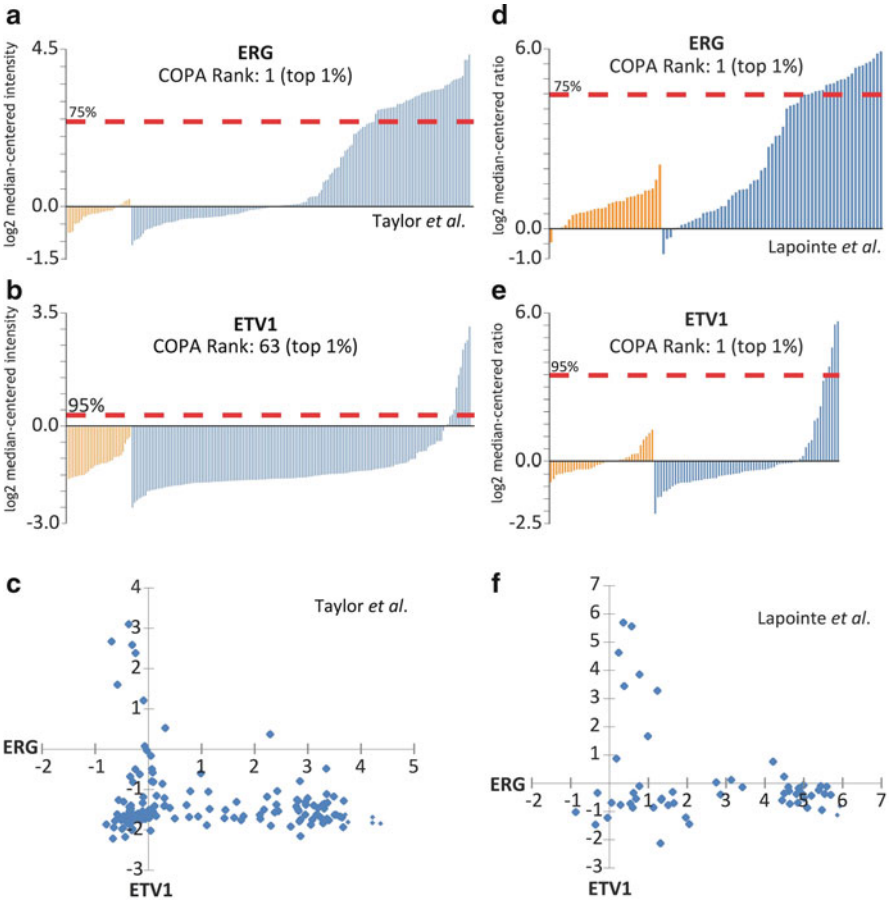

Fig. 5.1 *Cancer outlier profiling analysis (COPA) identifies the ETS family members ERG and ETV1 as high ranking outliers in prostate cancer gene expression profiling studies*. We developed COPA to identify genes showing marked overexpression in a fraction of tumors. Application of COPA to the Oncomine database (http://www.oncomine.com), a compendium of prostate cancer profiling studies, prioritized the ETS family members *ERG* and *ETV1* as outliers in prostate cancer [1, 6]. (**a**–**c**) Profiles for (**a**) *ERG* and (**b**) *ETV1* from the Lapointe et al. prostate cancer profiling study [21] were downloaded from Oncomine, which precomputes COPA scores for every study. Each *bar* represents gene expression in an individual benign prostate (*orange*) or prostate cancer (*blue*) sample. The COPA rank (of all genes in the study) for *ERG* or *ETV1 at* the indicated cutoff (75th, 90th or 95th percentile, *red dashed line*) is given. (**c**) Coexpression of *ERG* and *ETV1* demonstrates mutually exclusive outlier expression. (**d**–**f**) As in (**a**–**c**), except data from the Taylor et al. [22] prostate cancer profiling study

COPA prioritized two members of the ETS family, *ERG* and *ETV1*, as high ranking outliers in multiple prostate cancer profiling studies [1]. Strikingly, *ERG* and *ETV1* showed nearly mutually exclusive overexpression in all prostate cancer studies (i.e., a single tumor sample may overexpress *ERG* or *ETV1*, but never both), suggesting functional redundancy. The outlier profiles of *ERG* and *ETV1* in two prostate data sets, one included in our initial COPA analysis (Lapointe et al. [21]), and one from a recent profiling study (Taylor et al. [22]), and their coexpression, are shown in Fig. 5.1.

Given that homologues of this gene family are rearranged in Ewing's sarcoma (most commonly *FLI1*), we postulated that the mechanism of ETS gene overexpression in prostate cancer might similarly be due to rearrangement or amplification. Preliminary analysis of array comparative genomic hybridization (aCGH) data did not identify amplifications at the *ERG* locus. Thus, we performed exon-walking quantitative PCR, which demonstrated loss of expression of the 5′ exons in both *ERG* and *ETV1* in cases with outlier expression of the respective ETS family member. To determine the structure of the 5′ ends of *ERG* and *ETV1*, we performed 5′-RNA ligase-mediated rapid amplification of cDNA ends (5′-RACE), which unexpectedly identified replacement of the 5′ end of the ETS family member with sequences from the 5′ untranslated region of *TMPRSS2* [1]. Importantly, *TMPRSS2* is a strongly androgen regulated gene, providing an immediate mechanism for the marked overexpression of *ERG* or *ETV1*, where the 5′ promoter elements that normally drive androgen-mediated expression of *TMPRSS2* in the prostate now drive aberrant expression of *ERG* or *ETV1*. Unlike the *BCR*:*ABL1* gene fusion that encodes a chimeric kinase, ETS gene fusions encode a nearly full-length ETS transcription factor [6], and the fused transcript from *TMPRSS2* (or other 5′ partners) rarely encodes translated sequence. Thus, ETS gene fusions in prostate cancer are more functionally equivalent to *IGH* mediated overexpression of *MYC* in Burkitt's lymphoma (defined by the *IGH:MYC* fusion). Our initial discovery of ETS gene fusions identified *ERG* or *ETV1* rearrangements in more than half of all prostate cancers, which, given the prevalence of prostate cancer, makes ETS fusions the most prevalent gene fusion in cancer.

Diversity of ETS Gene Fusions Prostate Cancer

In serum prostate-specific antigen (PSA)-screened cohorts from Caucasian populations, approximately half of all prostate cancer foci harbor recurrent gene fusions of the androgen-regulated gene, *TMPRSS2*, to the oncogenic *ETS* transcription factor *ERG* (see below for detailed discussion). While *ERG* contributes to approximately 90 % of ETS family gene rearrangements in prostate cancer, other *ETS* transcription factors including *ETV1* [1], *ETV4* [23], *ETV5* [24], and *FLI1* [25] also participate in prostate cancer rearrangements, albeit at a lower frequency. Similarly, chimeric read-through transcripts involving neighboring genes *SLC45A3* and *ELK4*, resulting in *SLC45A3*:*ELK4* fusion transcripts, similarly occur in 5–10 % of prostate cancers [26, 27]. The structures of the various protein products encoded by all ETS genes and those involved in prostate cancer fusions are shown in Fig. 5.2a, b. Interestingly, the ETS genes rearranged in prostate cancer contain a highly conserved ETS DNA-binding sequence that is slightly divergent from other ETS proteins (Fig. 5.2c).

In contrast to the ETS gene family, which constitutes the most frequent 3′ partners, several different 5′ partners have been identified including those with androgen-responsive promoters (including *TMPRSS2*, *SLC45A3*, *KLK2*, *HERV-K_22q11.23* and *CANT1*), one with an androgen-insensitive promoter (*DDX5*), one

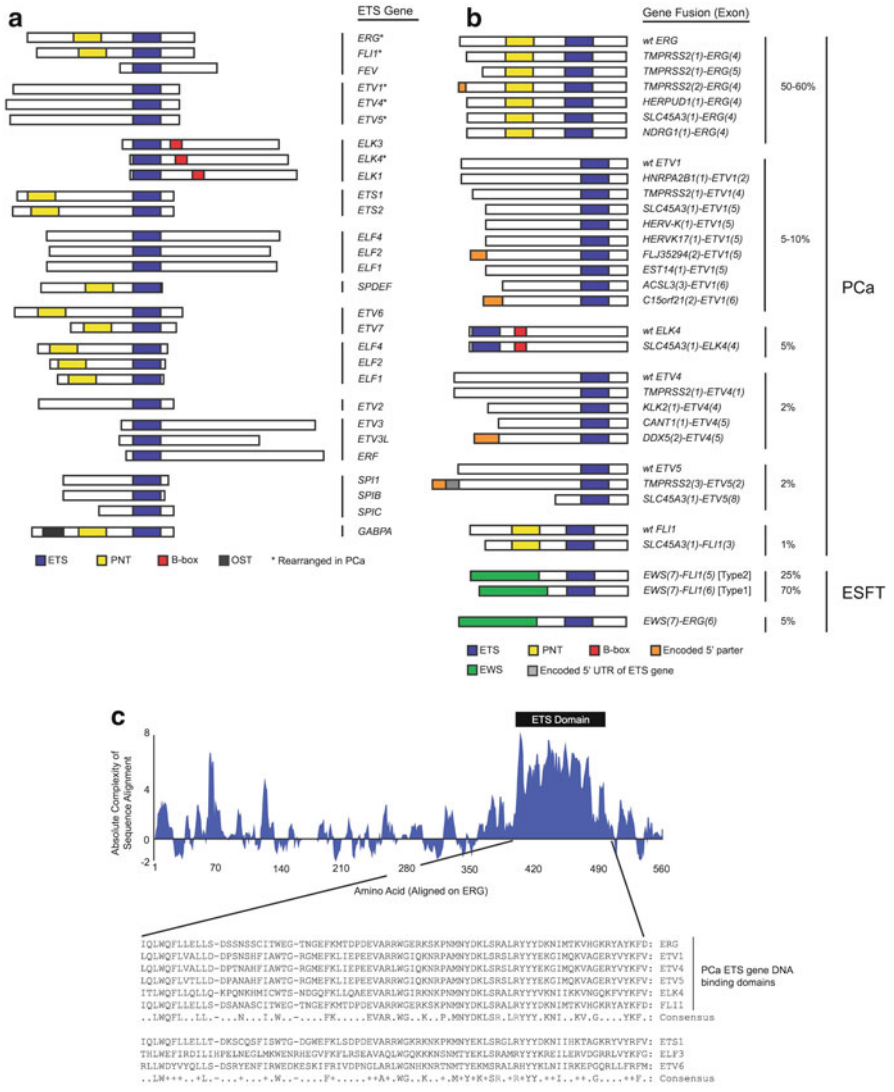

Fig. 5.2 *Schematic representation of conserved domains in ETS proteins and ETS gene fusion protein products*. (**a**) Schematic representation of the primary protein products of the 28 different ETS genes in humans. (**b**) Schematic representation of the protein products of the ETS gene fusions found in prostate cancer and Ewing's sarcoma. While most ETS gene fusions in prostate cancer drive slightly truncated ETS transcription factors, a few translate into chimeric proteins. (**c**) Amino acid sequence alignment of the ETS DNA binding domain from the ETS genes rearranged in prostate cancer (*ERG*, *ETV1*, *ETV4*, *ETV5*, *ELK4*, *FLI1*) and comparison with three that have not been observed as fusions in prostate cancer (*ETS1*, *ELF3*, *ETV6*)

with a constitutively active promoter (*HNRPA2B1*), and, in a single case, one with a highly expressed, but androgen-repressed promoter (*C15orf21*) [1, 23, 24, 28–30]. The reported spectrum of 5′ partners is certainly incomplete. For example Attard et al. found that only 8 of 23 (34 %) cases with *ETV1* rearrangements by FISH harbored 5′ partners known at that time and were able to identify *ASCL3* as a new 5′ fusion partner of *ETV1* [31]. Numerous rare novel 5′ partners will likely continue to be identified, particularly with the advent of RNA-seq, which allows for the identification of the 5′ end of gene fusions far more expediently than 5′-RACE or similar techniques. Nevertheless, *TMPRSS2:ERG* is by far the most prevalent ETS gene fusion observed in prostate cancer, likely due to the proximity of these two genes on chromosome 21 (approximately 3 MB apart), as described below.

In a recent IHC expression analysis study, we demonstrated that ERG protein expression was extremely high and at similar levels regardless of the 5′ fusion partner as fusions involving *TMPRSS2*, *SLC45A3* and *NDRG1* (to *ERG*) [32]. Although Perner et al. reported decreased expression of SLC45A3 protein expression in cases with *SLC45A3:ERG* gene fusions, ERG protein expression was not associated with the 5′ partner (*SLC45A3* or *TMPRSS2*). High expression of ERG regardless of the 5′ partner likely reflects the massive overexpression driven by androgen response elements combined with essentially zero background expression of ERG in benign prostate tissue or prostate cancer without rearrangements.

The most common *TMPRSS2:ERG* gene fusion variants involve *TMPRSS2* (NM_005656.3) exon 1 or 2 fused to *ERG* (NM_004449.4) exon 2, 3, 4, or 5 [1, 33–41] and less frequently rearrangements of *TMPRSS2* exon 4 or 5 fused to *ERG* exon 4 or 5 [39]. Additional variants have been reported, including those using novel *TMPRSS2* start sequences [37, 42], and various internal *ERG* exons may be present/absent, however, in our experience, the vast majority of *TMPRSS2:ERG* fusions involve exon 1 of *TMPRSS2* and 4 of *ERG* (also equivalent to exon 2 of *ERG* [NM_182918.3]). As such, the most prevalent *TMPRSS2:ERG* gene fusions encode for an ERG protein product that is missing only 4 N-terminal amino acids as compared to wild-type protein and, like *TMPRSS2* [43–45], the *TMPRSS2:ERG* gene fusion is androgen regulated [1]. Although groups have reported that expression of specific *TMPRSS2:ERG* splice variants have associations with cancer aggressiveness, signaling pathways, and phenotypic effects in model systems [41, 42, 46–48], their relevance to human cancers, where many splice isoforms are typically expressed, is unclear and large scale validation studies are needed. Importantly, as with the 5′ fusion partner, ERG protein expression also appears independent of the *TMPRSS2:ERG* fusion mRNA variant [32].

Prevalence of ETS Gene Fusions in Prostate Cancer

In the initial report of ETS gene fusions in prostate cancer, we reported *ERG* or *ETV1* rearrangements in 79 % of 29 prostate cancers by fluorescence in situ hybridization (FISH), in samples obtained from a PSA-screened radical prostatectomy cohort [1].

Subsequent studies have used a variety of techniques to assess the prevalence of ETS gene fusions in prostate cancer, including reverse transcription-PCR (RT), FISH, quantitative PCR (qPCR), and more recently, immunohistochemistry (IHC). As summarized by Kumar et al., initial studies in PSA-screened cohorts demonstrate ETS gene fusions in approximately 50 % of 1,500 cancers [5]. Similarly, we reviewed reports from an additional 10 studies comprised of 818 cases and identified an overall *TMPRSS2:ERG* gene fusion prevalence of 44 % [6]. Most recently, Pettersson et al. summarized ETS gene fusion prevalence (primarily rearrangements involving *TMPRSS2* and/or *ERG*) across published studies and reported a prevalence of 47 % across 10,779 cases [49].

Likewise, multiple studies assessing other ETS gene fusions have identified approximately 10 % of specimens showing *ETV1* > *ETV5* = *ETV4* > *FLI1* = *ELK4* rearrangements [23–25, 28–30, 36, 50–55]. Additionally, marked overexpression of *SLC45A3*:*ELK4* read-through transcripts have also been identified in ~10 % of prostate cancers, and although present at low levels in benign prostate tissues, the chimeric *SLC45A3:ELK4* fusion appears to have oncogenic functions similar to other ETS gene fusions [26, 27, 56–58]. Lastly, recent work has reported that ETS genes can be overexpressed without rearrangement and function similarly to ETS gene fusions [59]. Given that all known ETS gene fusions result in marked overexpression of the involved 3′ ETS gene, a simple estimate of the prevalence of ETS gene fusions can be made by assessing for ETS gene overexpression in microarray data, which usually shows mutually exclusive outlier expression of ETS genes known to be involved in gene fusions, as shown in Fig. 5.3.

The recent development of independent monoclonal antibodies directed against ERG [32, 64], which show utility for IHC on routine formalin fixed paraffin-embedded sections, has allowed for easier assessment of *ERG* rearrangement status than RT-PCR or FISH, and has allowed for the profiling of large cohorts. Importantly, both antibodies show high concordance with FISH for *ERG* rearrangement. Table 5.1 summarizes ERG immunohistochemistry results across prostate cancer studies utilizing biopsy and prostatectomy specimens and demonstrates an overall *ERG* gene fusion prevalence (indicated by positive ERG staining in cancer) of 48 % in 7,689 samples.

Caveats to Interpreting ETS Gene Fusion Prevalence Reports

Several important caveats to interpret ETS gene fusion prevalence across studies are needed, including the method of ETS gene fusion detection and the clinical and pathological characteristics of the cohort. The method of detection can dramatically influence the 5′ partners, 3′ ETS genes, and specific gene fusions detected. For example, RT-PCR is limited to only detecting fusion transcripts compatible with the utilized primers. After our initial report of *TMPRSS2:ETV1* fusions, we were struck by the lack of *TMPRSS2:ETV1* fusions detected by RT-PCR in follow-up studies (1 %) [33, 35, 39, 40, 53, 78, 79], yet *ETV1* was consistently overexpressed in

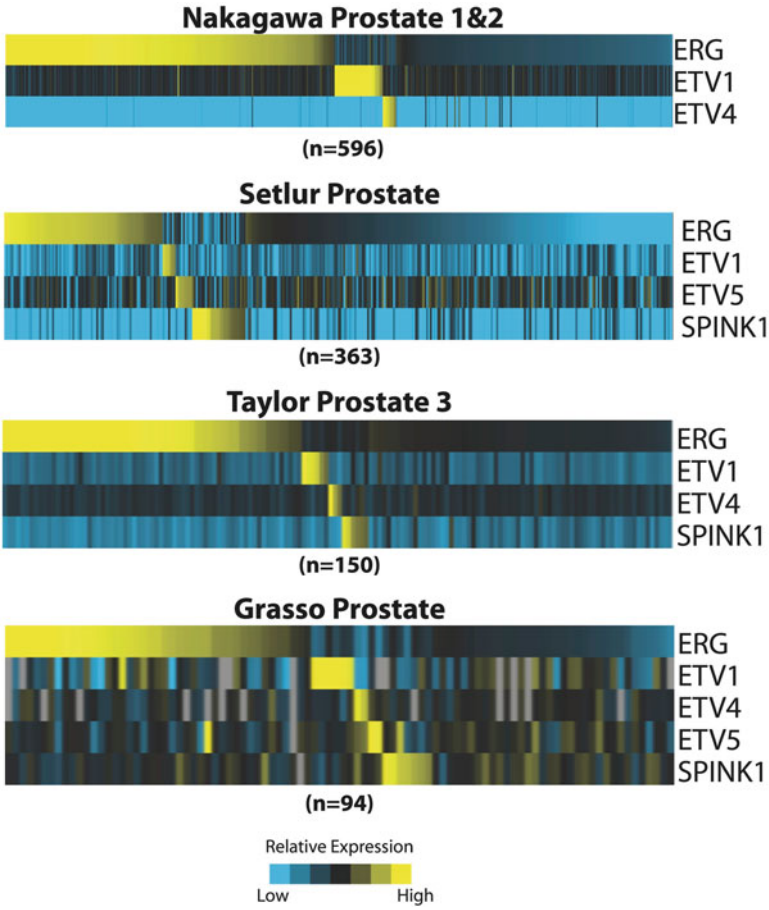

Fig. 5.3 *Outlier expression of ETS genes (ERG, ETV1, ETV4, and ETV5) and SPINK1 in prostate cancer microarray studies*. Gene expression data for the indicated ETS genes and *SPINK1* was downloaded from four prostate cancer profiling studies in the Oncomine database (http://www.oncomine.com), a compendium of cancer microarray studies. Heatmaps for all cancer samples in each study were generated using Cluster and TreeView on the Oncomine normalized data (*z*-score units). Studies are identified by the last name of the first author [22, 60–62] and the number of cancer samples in the study is indicated below the heatmap. *Yellow* and *blue* indicate relative over and under-expression, respectively, according to the legend. *Gray cells* indicate measurements not passing individual study filtering. Samples are ordered by outlier status [1, 63] of the indicated genes

approximately 6–16 % of gene expression microarray studies. Thus, we utilized RACE to characterize cases with *ETV1* overexpression and identified numerous 5′ partners involved in recurrent rearrangements with *ETV1* [54]. More recently, the advent of immunohistochemistry for ERG, as a surrogate for *ERG* rearrangements, has simplified the assessment of large tissue cohorts. However, such studies do not assess other ETS family members, where specific antibodies against *ETV1*, *ETV5*, *ETV4* and *ELK4* are needed.

Table 5.1 ERG protein expression in prostate cancer by immunohistochemistry (IHC)

First author	Study	Sample type	Samples (*n*)	ERG$^+$ (*n*)	ERG$^+$ (%)	Antibody	FISH samples (*n*)	IHC/FISH concordance
Park	[32]	RRP	207	92	44	EPR3864	207	>95 % Sens. & Spec.
van Leenders	[65]	RRP/Bx	124	81	65	EPR3864	NA	NA
Yaskiv	[66]	RRP/Bx	77	32	42	EPR3864	NA	NA
Chaux	[67]	RRP	427	192	45	EPR3864	427	86 % Sens., 89 % Spec.
Furusato	[68]	RRP (Asian)	209	42	20	EPR3864	NA	NA
Falzarano	[69]	RRP	305	100	33	EPR3864	305	>95 % Sens. & Spec.
Minner	[70]	RRP	2,805	1,469	52	EPR3864	453	>95 % Sens. & Spec.
Hoogland	[71]	RRP	437	239	55	EPR3864	NA	NA
Braun	[72]	RRP	278	118	42	EPR3864	278	>95 % Sens. & Spec.
Pettersson	[49]	RRP	1,180	584	49	EPR3864	NA	NA
Perner	[73, 74]	RRP	630	282	55	EPR3864	630	95 % Sens., 87 % Spec.
Tomlins	[75]	Bx	160	71	44	EPR3864	NA	NA
Furusato	[64]	RRP	261	117	45	CPDR ERG	10	100 % Concord.
Miettinen	[76]	RRP/ TURP	66	30	45	CPDR ERG	NA	NA
Braun	[72]	RRP	278	120	43	CPDR ERG	278	>95 % Sens. & Spec.
Kron	[77]	RRP	245	125	51	CPDR ERG	NA	NA
		Total:	**7,689**	**3,694**	**48**			

Studies are identified by the last name of the first author. Studies reporting the first characterization of the indicated ERG antibodies are italicized. Sample type, including prostatectomy (RRP), biopsy (Bx) and transurethral resection (TURP) are indicated. The number of samples evaluated, the number and percent ERG positive, and the antibody used are indicated. If FISH was performed, the number of samples assessed and the IHC/FISH concordance, or sensitivity (Sens.) and specificity (Spec.) is given.

Another important caveat for interpreting ETS gene fusion prevalence is the method of prostate cancer detection, which is often related to the presence of PSA screening. Importantly, prior to widespread PSA screening, prostate cancer was commonly detected by transurethral resection (TURP) for relief of urinary symptoms. More recently, the advent of PSA screening has resulted in the vast majority of cancers being detected by needle biopsy. Furthermore, TURP obtains tissue predominantly from the transition zone (more central), while needle biopsies are directed toward the peripheral zone. Hence, cancers detected by TURP may

represent exclusively transition zone cancers or direct extension from peripheral zone tumors. Hence, these factors can dramatically influence the evaluation of ETS gene fusion prevalence.

Although ETS gene fusions have been consistently identified in ~50 % of prostatectomy or needle biopsy specimens from PSA-screened cohorts, TURP-based studies from non-PSA screened cohorts (population cohorts) have consistently shown lower ETS gene fusion prevalence. Three large population-based cohorts reported the prevalence of *ERG* gene fusions to be 15 % (including clinical stage T1a, b), 18 % (T1a, b), 30 % (T1–3), and 35.5 % (T1–3) [60, 80–82]. Importantly, in studies where staging information was provided, clinical T1 stage cancers had the lowest *ERG* gene fusion prevalence (15 and 17 %) [80, 81], and in the study by Setlur et al., 10 % of patients with <=5 % of tissue containing cancer had *ERG* rearrangements, while 31 % of patients with >50 % tissue containing cancer had *ERG* rearrangements [60]. As T1 cancers are more likely to be exclusively transition zone cancers, this suggested that transition zone cancers may be less likely to harbor ETS gene rearrangements. In 2009, Guo et al. studied 30 radical prostatectomy cases with multifocal prostate cancer, including at least one transition zone focus. By FISH, although 43 % of peripheral zone tumor foci harbored *ERG* rearrangements, no transition zone foci harbored *ERG* rearrangements [83]. In 2010, Falzarano et al. identified dominant transition zone cancers in 62 of 397 (16 %) of radical prostatectomy specimens and 46 of the 62 tumors harbored a secondary peripheral zone tumor focus. By FISH, they identified *ERG* rearrangements in 12 and 34 % of transition and peripheral zone tumors, respectively [84]. Additional studies have similarly found a low frequency of ERG rearrangements in T1 tumors or transitional zone tumors [85, 86]. Taken together, the available data demonstrate that transition zone tumors have lower rates of ETS gene fusion (at least with respect to *ERG*) than peripheral zone tumors, suggesting that distinct genetic alterations drive tumors in these zones.

Another important caveat of interpreting ETS gene fusion prevalence is the ethnicity/race of the cohort studies. In their study of patients undergoing diagnostic biopsy in the USA, Mosquera et al. reported that 44 of 85 (52 %) Caucasian patients had *ERG* rearrangements, while only 2 of 15 (13 %) non-Caucasian patients had *ERG* rearrangements [87]. Subsequently, Magi-Galluzi et al. reported *ERG* rearrangements in 21/42 (50 %) of Caucasian and 20/64 (31 %) of African-American patients undergoing prostatectomy in the USA, but only 7/44 (16 %) of Japanese patients undergoing prostatectomy in Japan [88]. Similarly, Miyagi et al. reported *TMPRSS2:ERG* gene fusions by RT-PCR in 54 of 194 (28 %) of 194 Japanese radical prostatectomy specimens [58]. Mao et al. found *ERG* rearrangements in 64 of 155 (41 %) predominantly Caucasian patients undergoing prostatectomy in the UK, but only 7 of 93 (8 %) Chinese prostatectomy specimens [89]. Together, these data suggest that prostate cancers arising in patients of different ethnicity/race are driven by differing combinations of molecular alterations. Evaluation of additional subtypes of prostate cancer in diverse populations will likely be required to fully understand unique populations of prostate cancer that may be amendable to specific therapies.

Genesis of ETS Gene Fusions

ETS gene fusions occur early in prostate cancer progression during the transition from high-grade prostatic intraepithelial neoplasia (PIN) lesions to invasive carcinoma [90]. Consistent with this observation, we demonstrated that ERG IHC staining occurred exclusively in prostate cancer and adjacent HGPIN (see below for further discussion [32]). Importantly, isolated ERG positive (rearranged or protein expressing) high grade PIN, meaning PIN distant to cancer, is rarely seen in prostatectomy specimens [64, 91, 92], suggesting that *ERG* rearrangements lead to the development of cancer, rather than serve as a dead end lesion. If ERG rearrangements led to dead end lesions, isolated *ERG* rearranged HGPIN should accumulate during aging.

Two rearrangement mechanisms have been described for the formation of ETS gene fusions (reviewed in [93]). Because the *TMPRSS2* and *ERG* genes are located 3 MB apart on chromosome 21q, rearrangement can occur by either interstitial deletion [36, 40, 53, 78] or by interchromosomal insertion [94]. Recently, several groups demonstrated that androgen stimulation causes an induced chromosomal proximity of the *TMPRSS2* and *ERG* genomic loci in cell lines [95–97], which can lead to the formation of *TMPRSS2:ERG* rearrangements at low frequency [95, 96]. Importantly, with the addition of genomic stress such as ionizing radiation, the frequency of fusion formation increases dramatically [96, 97]. Lin et al. postulated that endogenous endonucleases including activation-induced cytidine deaminase (AID) and the protein encoded from LINE-1 ORF2 are responsible for creating AR-directed DNA double strand breaks that are required for site-specific genomic rearrangements [96]. In line with this hypothesis, studies of translocations following site-directed DNA double strand breaks suggest that gene fusions preferentially utilize actively transcribed genes [98, 99]. Consequently, Berger et al. demonstrated that the fusion breakpoints in prostate cancer overlap with histone markers associated with open chromatin as well as AR binding in tumors with ETS rearrangements [100]. In summary, data suggest that ETS gene fusions are formed by an AR-dependent mechanism lending support to the observation that *TMPRSS2:ERG* gene fusions are prostate specific.

Functional Role of ETS Gene Fusions in Cancer Development

Subsequent to gene fusion formation, ETS gene fusions have a clear role in prostate cancer pathogenesis. Genetically engineered mice expressing *ERG* or *ETV1* under androgen regulation exhibit PIN-like lesions, but do not develop frank carcinoma [5, 54, 101–105], and overexpression of *ERG* leads to accelerated prostate carcinogenesis in some backgrounds when combined with deletion of the tumor suppressor *PTEN* [102, 103, 105]. Additionally, in a transplant model, mouse prostate epithelial cells that are forced to overexpress both *ERG* and the androgen receptor (*AR*) gene form invasive prostate cancer [104]. This suggests that *ERG* accelerates prostate carcinogenesis, although predicting temporal events in human prostate cancer from mouse models is challenging. Furthermore, because these models do not develop

advanced disease, the data suggest that either a fundamental difference between mice and men exists—especially considering that wild type mice rarely if ever develop prostate cancer—or that we do not yet have a complete understanding of all of the disruptive events that occur prior to metastatic progression in ETS positive tumors.

Functionally, ETS gene expression drives several malignant phenotypes and is critical for the survival of cancer cells. In VCaP cells, which were derived from a vertebral metastatic prostate cancer lesion and naturally harbor the *TMPRSS2:ERG* rearrangement [106], *TMPRSS2:ERG* targeting siRNA inhibits the ETS transcriptional program, cell growth, invasion, metastasis, and xenograft tumor growth [47, 107]. Likewise, forced overexpression of truncated *ERG* or other ETS genes, such as *ETV1*, *ETV4* and *ETV5*, which are commonly rearranged in prostate cancer, in benign immortalized or nontransformed prostate epithelial cells, drives a unique ETS transcriptional program, which in turn drives cell invasion [1, 23, 24, 108]. Taken together, ETS gene fusions represent a prostate cancer-specific genetic lesion that has promise as a target for molecularly tailored therapy.

ETS gene fusions induce DNA double strand breaks in prostate cancer [100]. Although the mechanism is unknown, the consequences of the DNA damage may help to explain the relatively slow progression of prostate cancer even after genomic rearrangement. Double strand breaks in DNA are generally repaired by one of two repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). While HR uses a complementary strand of DNA to execute an error-free repair, the NHEJ pathway ligates together the two broken ends of DNA following processing on non-compatible ends. As such, DNA at the site of double strand breaks will acquire mutations and deletions as a direct result of the NHEJ repair mechanism. This is functionally important because it could explain why some prostate cancers remain indolent for many years only to spontaneously become highly aggressive after acquiring a secondary disruptive event. Some therapeutic strategies have been developed to take advantage of the fact that ETS gene fusions lead to increased DNA damage in cells (see below).

ETS Gene Fusions as the Basis for the Molecular Suptyping of Prostate Cancer

Given their extraordinary prostate cancer specificity, ETS gene fusions represent an obvious biomarker and potential therapeutic target in prostate cancer. In addition, as they are not present in all prostate cancers, they have the potential to serve as the basis for subtyping prostate cancers. However, as the main goal of molecular subtyping is to personalize cancer detection and treatment (so called “personalized medicine”), the full context of driver events in the genesis and progression of cancer must be considered. Additionally, prostate cancer has several unique aspects, such as nearly ubiquitous multifocality, where a single patient’s prostate may harbor two or more spatially and molecularly distinct cancer foci (multifocal disease). The advent of DNA microarrays and next generation sequencing has allowed the rapid profiling of driver events in prostate cancer. Together with studies using single gene

approaches, these methods have confirmed that the two most prevalent focal somatic alterations in prostate cancer are ETS rearrangements [1] and disruptions in the tumor suppressor gene *PTEN*, which lead to activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway [109]. As described below, while ETS gene fusions and *PTEN* status can be used to form a simple molecular stratification system ($ETS^{+/-}/PTEN^{+/-}$), the identification of additional disruptive events such as those in the *SPINK1*, *RAF/RAS*, *CHD1*, and *SPOP*, is now enabling further sub-stratification of the disease. Here, we will discuss these additional abnormalities and how they can be used to sub-stratify prostate cancer.

ETS Gene Fusions to Evaluate Prostate Cancer Multifocality

Prior to discussing the molecular subtyping of prostate cancer, the multifocality must be taken into account, as this significantly affects the interpretation of nearly all biomarker studies in prostate cancer. Histopathologic examination of prostate cancers at prostatectomy nearly always demonstrates spatially and morphologically distinct foci of cancer, which are often of varying size and Gleason grade, suggesting that these are unique cancer foci (multifocality). Estimates of multifocality range up to 80–90 % [110, 111], with unifocal tumors consisting predominantly of minute low Gleason grade foci or very large aggressive foci that have likely "overrun" the remaining prostate. Hence, as the majority of biomarker studies in prostate cancer cannot assess all foci, a single focus, typically the largest and histologically most aggressive focus (which is not always equivalent), is selected for evaluation. Although in some cases identification of the most aggressive focus is easy, PSA screening has led to the detection and resection of prostates containing small to medium-sized foci of similar Gleason score. Assuming that the largest, or most histologically aggressive focus as the time of resection is the one that will later cause poor outcome (such as PSA recurrence or metastasis), is fraught with assumptions in the absence of clear aggressiveness (such as extraprostatic extension).

ETS gene fusions are nearly perfect clonal markers, since if present in a focus, they are present in nearly every cell, as shown by both FISH and IHC. Furthermore, ETS gene status does not change in prostate cancer progression, as supported by several lines of evidence. Both localized and lethal, heavily treated, chemotherapy- and castration-resistant prostate cancer (CRPC) show approximately 50 % ETS gene fusion prevalence, unlike *PTEN* deletions, which increase with progression ([29, 61] and reviewed in [6]) . For example, Mehra et al. demonstrated uniform ETS status among multiple foci of metastatic cancer from the same patient, i.e., either all or no metastatic deposits harbor the same ETS gene fusion [29]. Similarly, Attard et al. found that when the primary tumor (thought to lead to the metastatic lesion) harbored an *ERG* rearrangement by FISH, all circulating tumor cells (circulating tumor cells) from the patient harbored *ERG* rearrangements, while other events, such as *PTEN* deletion or *AR* amplification, were heterogeneous between CTCs [112].

Thus, although multifocal tumors have long been assumed to be genetically distinct, which has been supported by single locus FISH and microsatellite-based

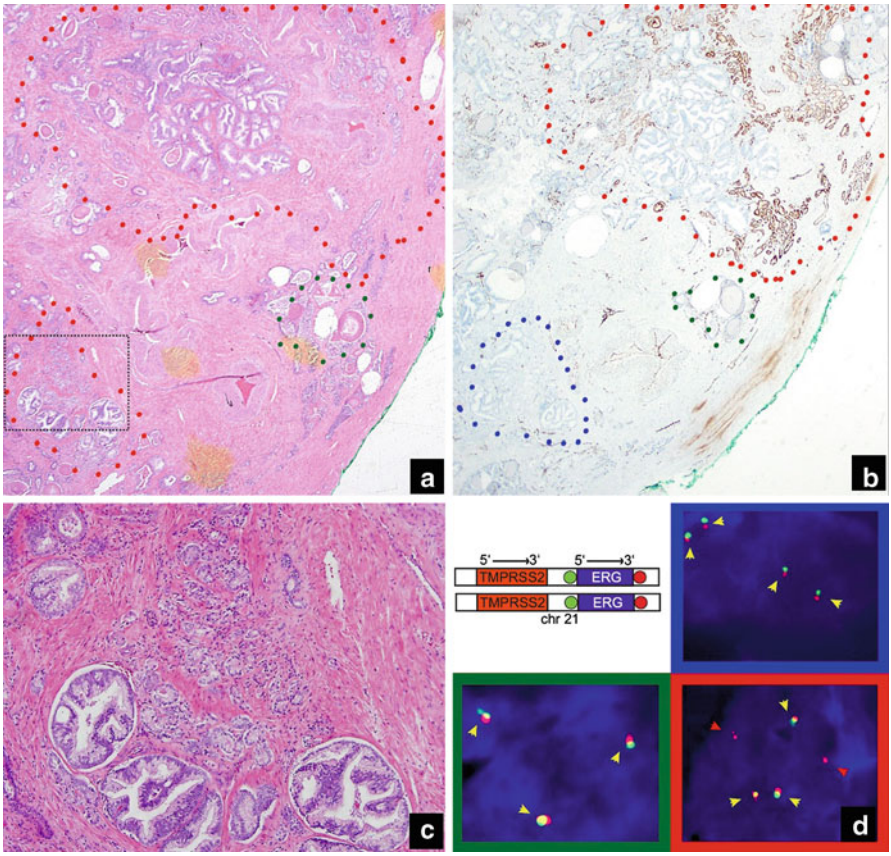

Fig. 5.4 *Collision of ERG+ and ERG− negative tumor foci by combined immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH).* (**a**–**c**)**.** A prostatectomy section with what was thought to be a single focus of carcinoma (**a**, outlined by *red*), was stained for ERG by IHC (**b**) and demonstrated distinct ERG$^+$ (outlined by *red*) and ERG$^-$ (outlined by *blue*, higher magnification shown in **c**) foci. (**d**) FISH performed using split probes flanking ERG (schematic shown in *upper left panel* of **d**), demonstrated no *ERG* rearrangement (all *yellow* signals) in the ERG$^-$ foci (*blue*) and benign prostatic glands (*green* and outlined in **a** and **b**), while the ERG$^+$ foci contained a deletion of the 5′ ERG signal (*green*), consistent with *ERG* rearrangment through deletion. Orginial magnifications 2.5× (**a**, **b**), 10× (**c**), and 100× (**d**)

studies [110, 113–116], ETS status provides a simple measurement that can determine whether multifocal tumors arise from distinct clones. As reviewed in [6], multiple groups have now shown that while individual foci are clonal with respect to ETS status (present or absent), 41–67 % of cases harbor individual cancer foci that differ with regard to the presence of ETS fusions or fusion mechanism (i.e., *TMPRSS2:ERG* fusions through deletion or insertion) [34, 117–119]. An example of a collision of ERG$^+$ and ERG$^-$ clones of what was thought to be a single focus of prostate cancer, which showed intrafocal concordance of FISH for *ERG* rearrangement and IHC for ERG expression, is shown in Fig. 5.4. Thus, studies

have now begun to analyze patterns of ETS gene fusions in primary and matched metastatic tumors that may allow one to predict which focus will metastasize or recur at the time of diagnosis [55, 73, 120, 121].

ETS Status Defines Unique Molecular Subtypes of Prostate Cancer (ETS$^+$ vs. ETS$^-$)

Given the profound effects of in vitro recapitulation of ETS gene fusions and their early and clonal role in prostate cancer, it is logical that ETS status would define distinct molecular subtypes of prostate cancer. Importantly, several studies have now demonstrated that ETS$^+$ vs. ETS$^-$ cancers are indeed distinct molecular subtypes. For example, we compared the DNA microarray signature of prostate cancers with or without overexpression of *ERG*, *ETV1* or *ETV4* (as a surrogate for ETS fusions) to other prostate cancer studies using the Molecular Concepts Map in the Oncomine database and demonstrated that ETS overexpressing signatures were highly enriched [21, 122, 123]. Similarly, using complementary DNA-mediated annealing, selection, ligation, and extension (DASL) profiling of gene expression from 354 patients, Setlur et al. identified an 87-gene expression signature that was highly accurate at distinguishing *TMPRSS2:ERG* positive from negative prostate cancers in an independent cohort of 101 patients (AUC = 0.80) [60].

To further demonstrate the robustness of ETS$^+$ vs. ETS$^-$ gene fusion positive vs. negative prostate cancers as distinct molecular subtypes, we used Oncomine (http://www.oncomine.com) to identify the gene expression signature comprised of the top 1 % of genes overexpressed in *TMPRSS2:ERG* fusion positive vs. negative cancers from the 354 patient cohort in the Setlur et al. gene expression profiling study. Oncomine precomputes enrichment (based on disproportionate overlap) with over 10,000 other molecular concepts, including gene expression signatures, clustered gene expression, literature-based concepts, and standard biological annotation (i.e., GO terminology) [124, 125]. As shown in Fig. 5.5, the Setlur et al. *TMPRSS2:ERG* gene fusion signature identified a highly connected network of molecular concepts related to prostate cancer, including multiple ETS gene fusion signatures. When compared to all other concepts in the Oncomine database, the Setlur et al. *TMPRSS2:ERG* gene fusion signature shared the most significant overlap with the signature of *TMPRSS2:ERG* gene fusion positive prostate cancers in the other cohort of 101 patients in the Setlur et al. study ($p = 6.88 \times 10^{-24}$, $q = 6.29 \times 10^{-19}$, odds ratio = 21). Importantly, the next most significantly enriched concept with the Setlur et al. *TMPRSS2:ERG* gene fusion signature was the signature of ETS gene fusion positive prostate cancers in the Grasso et al. [61] prostate cancer profiling study ($p = 6.01 \times 10^{-17}$, $q = 1.10 \times 10^{-12}$, odds ratio = 10.9). Taken together, these data demonstrate that ETS rearrangement status defines robust gene expression signatures, which when combined with data described below, demonstrates the utility of ETS status as the basis for prostate cancer molecular subtyping.

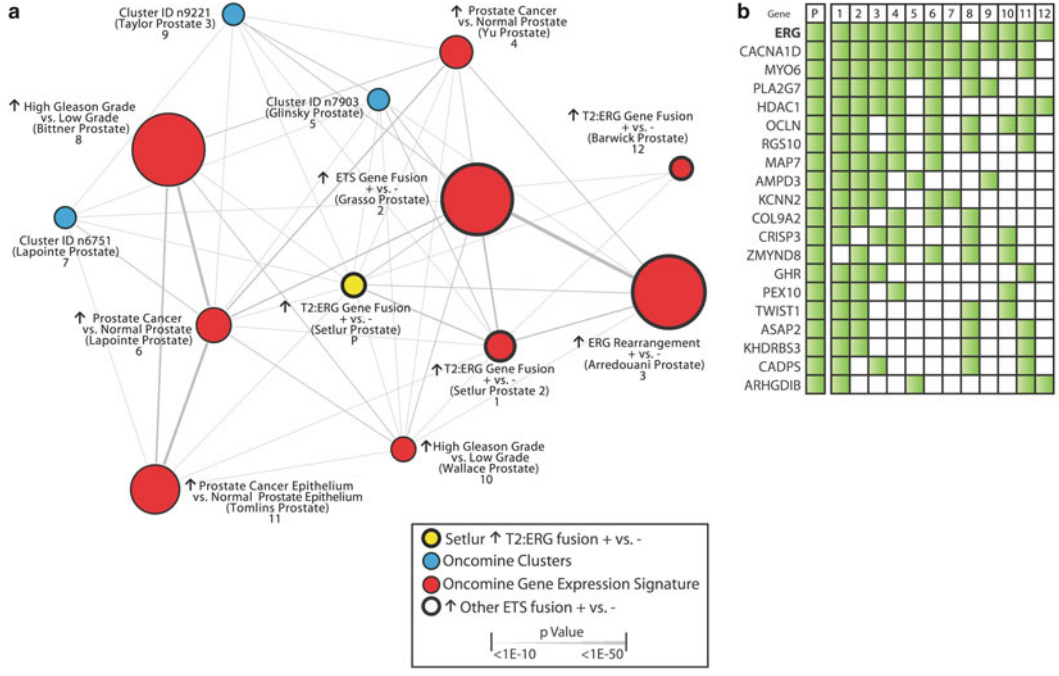

Fig. 5.5 *Molecular concepts mapping confirms ETS gene fusion positive and negative prostate cancers are distinct molecular subtypes.* (**a**) Molecular concepts mapping was performed using the Oncomine database [123–125] and visualized using Cytoscape. The signature of the top 1 % of genes overexpressed in *TMPRSS2(T2):ERG* gene fusion positive (+) vs. negative (−) prostate cancers from the Setlur et al. study [60] was used as the primary concept (*yellow node*). Oncomine precomputes significant enrichment against >10,000 other molecular concepts, including other gene expression signatures (*red*), significantly correlated gene clusters (*cyan*), literature concepts, and biological annotation. Selected concepts showing significant enrichment are visualized, with node size correlated to the number of genes in the concept, and the edge thickness indicating the significance of the enrichment according to the scale. ETS gene fusion+vs. − concepts are shown with *bold* rings. Only enrichments with an odds ratio >2 and p value $<1 \times 10^{-4}$ are shown. (**b**) Genes were ranked according to being present in the most number of visualized concepts, and the 25 top ranking genes are shown (*green* indicates present in that concept). Studies are indicated according to the numbers shown in (**a**)

ETS Positive, PTEN Deficient Prostate Cancer (ETS$^+$, PTEN$^-$)

As described above, disruption of *PTEN* (either through mutation or deletion) is one of the most common aberrations in prostate cancer. Overexpression of *ERG* in mice genetically engineered to have *PTEN* loss, leads to accelerated carcinogenesis [102, 103]. Additionally, in a transplant model, mouse prostate epithelial cells forced to overexpress both *ERG* and activated AKT form invasive prostate cancer as did mice expressing *ERG* and *PTEN* shRNA suggesting a functional role for *ERG* in *PTEN*$^{+/-}$ driven transformation in mice [104].

Intriguingly, several studies using multiple techniques for assessing *PTEN* and ETS genes fusion status (i.e., FISH, IHC, and aCGH) have now demonstrated that these lesions significantly co-occur [51, 61, 89, 91, 102, 103, 126–130]. For example, Minner et al. evaluated a large radical prostatectomy cohort and assessed ERG (by IHC) and *PTEN* (by FISH) status in 2,177 tumors [128]. They found that *PTEN* deletion was strongly associated with *ERG* rearranged cancers (29 % *PTEN* deletion in *ERG* rearranged vs. 11 % in *ERG* wild type tumors, $p < 0.0001$).

Similar to ETS status, where *ERG* rearrangements comprise the majority of lesions but a number of other ETS genes are involved less frequently, comprehensive characterization of prostate cancer has identified additional aberrations in the *PTEN* pathway that occur in prostate cancer. For example, through whole genome sequencing, Berger et al. identified recurrent disruption of *MAGI2*, which encodes a PTEN interacting protein, in two of seven prostate cancers [131]. Similarly, in an exome-sequencing study, Grasso et al. found that *PTEN* was directly disrupted in 48 % of CRPCs, while an additional 33 % had disruptions in genes encoding direct PTEN interactors, including a mutation in *MAGI2* and recurrent mutations in *MAGI3* [61]. Hence, evaluation of ETS/*PTEN* status may need to account for alternative ways to inactivate *PTEN* and downstream targets.

Common Aberrations in ETS Negative Prostate Cancer

SPINK1 (ETS$^-$/SPINK1$^+$)

Our initial application of COPA, which identified *ERG* and *ETV1* as high ranking outliers across multiple prostate cancer studies, was limited to known causal cancer genes ranking in the top 10 of any study. Hence, in an effort to comprehensively identify outliers specifically in prostate cancer, we utilized a meta-COPA approach across all prostate cancer studies in Oncomine to identify additional high ranking outliers. Importantly, this approach ranked both *ERG* and *ETV1* as top 10 meta-outliers across studies. Among the remaining candidates, we focused on *SPINK1* (*serine peptidase inhibitor, Kazal type 1*), which showed outlier expression in approximately 10 % of all prostate cancers and was not overexpressed in benign prostate tissue [63]. Strikingly, *SPINK1* overexpressing tumors were mutually

exclusive from those overexpressing *ERG* or *ETV1* across profiling studies. Through a combination of in vitro and in silico studies of ~1,800 prostate cancer, we confirmed that *SPINK1* outlier expressing tumors are mutually exclusive from tumors harboring ETS gene fusions, thus, defining a unique molecular subtype of the disease (ETS$^-$/*SPINK1*$^+$).

SPINK1 encodes a secreted 56-amino acid protein that is normally expressed in pancreatic acinar cells and is thought to protect the pancreas from autodigestion by preventing premature activation of proteases [132]. Functional studies of 22RV1 cells, which endogenously overexpress *SPINK1*, demonstrated that SPINK1 expression is required for cell proliferation, invasion, and xenograft growth [133]. Additionally, SPINK1 was shown to bind to the EGFR receptor and activate EGFR signaling in the absence of EGF ligand. Interestingly, antibody based therapy against either SPINK1 or EGFR significantly attenuated 22RV1 xenograft growth and additive in combination. As such, anti-SPINK1 and anti-EGFR therapies may have therapeutic benefit in this subset of ETS$^-$ prostate cancer.

CHD1 (*ETS*$^-$, CHD1$^-$)

Genomic sequencing studies have demonstrated that *CHD1*, which encodes an ATP-dependent chromatin-remodeling enzyme, is lost in approximately 5 % of all prostate cancers through copy number loss or somatic mutation and defines a unique molecular subtype from ETS$^+$ disease [61, 131, 134, 135]. Functional studies have demonstrated that RNAi mediated knockdown of *CHD1* is not sufficient for malignant transformation of normal cells or for changes in cell proliferation of benign immortalized prostate epithelial cells [134, 135]. However, *CHD1* knockdown was sufficient to drive cell invasion (mirroring the similar phenotype mediated by ETS genes in vitro) and led to increased clonogenicity in some cell line models [134].

SPOP (*ETS*$^-$, SPOP^{mut})

Recently, next generation sequencing of prostate cancer genomes led to the identification of recurrent mutations in *SPOP* (*speckle-type poxvirus and zinc finger protein*) [61, 126, 131, 136] in prostate cancer. *SPOP* encodes an adapter protein that localizes to nuclear speckles [137] and contains two conserved domains (Meprin and TRAF homology [MATH] and Bric-a-brac, Tramtrack, Broad complex [BTB]). Through the BTB domain, SPOP forms a complex with Cul3 (cullin3), a RING-H2-type E3 ubiquitin ligase, which ubiquitinates proteins binding to the MATH domain of SPOP for subsequent degradation [138]. Importantly, all identified *SPOP* mutations in prostate cancer occur in the substrate binding cleft and include residues known to be involved in substrate interaction. Barbieri et al. reported *SPOP* to be the most frequently mutated gene in their study of 112 prostate cancer exomes and confirmed the presence of *SPOP* mutations in 6–15 % of localized prostate cancers and CRPCs across more than 300 samples, while no mutations were identified in 41

benign prostate tissue samples [126]. Additionally, they demonstrated that either *SPOP* knockdown or expression of the most common variant (F133V) results in increased prostate cancer cell invasion, without significantly effecting cell growth. Similar to *SPINK1*, all reported prostate cancer foci with *SPOP* mutations lack ETS gene fusions across independent cohorts, supporting (ETS^{-}/*SPOP*^{mut}) as a distinct subtype of prostate cancer [61, 126].

Taken together, although the molecular mechanisms by which these newly discovered disruptive events drive prostate cancer progression are still largely unknown, it is clear that each plays a driving role in prostate cancer. Importantly, studies are now beginning to address the co-occurrence of these lesions, which should allow for a robust molecular classification of prostate cancer. For example, although *SPINK1* overexpression, *CHD1* aberrations, and *SPOP* mutations occur essentially only in ETS negative tumor foci, these events themselves are not mutually exclusive [61, 126]. Additionally, high throughput characterization of prostate cancer is beginning to identify additional events that may co-occur or are exclusive of ETS gene fusions. For example *TP53* disruption (by mutation or copy number loss) have been found to be enriched in ETS^{+} tumors [61, 126], while RAS/RAF family fusions and rearrangements, although rare in prostate cancer, have only been reported in ETS^{-} tumors [61, 126, 139, 140]. ETS-based stratification as the foundation of prostate cancer molecular subtyping has important implications for both biomarker and therapeutic targeting of prostate cancer, and we believe that molecular stratification of prostate cancer will soon become the standard of care.

Clinical Utility of ETS Gene Fusions

Given their prostate cancer specificity, ETS gene fusions have enormous promise as biomarkers. However, qualification for use in clinical management requires well-planned studies, and careful attention must be paid to replicating the clinical scenario where the biomarker would be used. Here we will review progress toward clinical application of ETS gene fusions as prostate cancer biomarkers in the area of tissue-based diagnosis, early detection, and risk stratification. An important caveat of ETS gene fusions is that they only occur in ~50 % of prostate cancer foci, which immediately suggests sensitivity limitations. However, as described above, prostate cancer is multifocal more often than not, and as ETS fusion status can be heterogeneous between foci, this often precludes the assignment of a man as harboring ETS^{+} or ETS^{-} prostate cancer when the index (biologically driving) focus is in doubt.

ETS Gene Fusions as Prostate Cancer Specific Biomarkers

As described above, ETS gene fusions, as detected by methods that are applicable to routine clinical practice (such as FISH and IHC), are incredibly cancer specific. By FISH, the vast majority of studies report no rearrangements in benign prostate

tissue. Similarly, IHC-based studies have rarely reported staining in benign glands. For example, Perner and Esgueva et al. reported that in tissue cores from 640 prostate cancers evaluated by FISH for *ERG* rearrangement and IHC for ERG expression, no benign prostate glands evaluated by either method were *ERG* positive [73, 74]. Similarly, two studies using whole mount prostatectomy specimens or quartered prostatectomy sections, with independent antibodies against ERG, reported >99.99 % specificity of positive staining for prostate cancer [64, 141]. In our study, we evaluated 169 quartered prostatectomy sections, containing a mixture of benign and cancerous elements (sections were selected to determine ERG status in all tumor foci present in the prostatectomy specimen), and found only ~35 ERG^+ benign glands in 8 cases [141].

The ideal biomarker in prostate cancer (100 % sensitive and specific) would need the following characteristics: easy applicability and evaluation on routine needle biopsy tissue specimens, homogeneous expression in cancer, no staining in benign glands or benign lesions that morphologically resememble prostate cancer, no staining of HGPIN, and the ability to add to/replace current diagnostic markers (most commonly AMACR, which is typically overexpressed in cancer, and basal cell markers, which are typically lost in prostate cancer). Importantly, ETS gene fusions have been evaluated in regard to most of these characteristics; however, there are important considerations when evaluating reported studies.

Both FISH and IHC are routinely applied to diagnostic tissue biopsy specimens, and both techniques have been applied to prostate cancer biopsy specimens. Mosquera et al. evaluated 134 prostate needle biopsy tissue specimens by FISH for *ERG* rearrangements and found that 46 of 100 (46 %) biopsies with cancer harbored *ERG* rearrangements, while 0 of 34 benign biopsies harbored *ERG* rearrangements, and benign glands in cancerous biopsies were similarly negative for *ERG* rearrangements [87]. Importantly, 26 cores were evaluated between GU pathologists at two institutions with 100 % agreement on *ERG* rearrangement status. Similarly, we recently evaluated ERG staining by IHC in biopsies from a retrospective cohort ($n = 111$), enriched in cores requiring diagnostic immunohistochemistry, and a prospective cohort from all cases during 3 months ($n = 311$) [75]. Among 418 evaluable cores, ERG staining was present in cancerous epithelium on 71 of 160 cores (44 %), with ERG expression in only 2 of 162 cores (1 %) diagnosed as benign. In total, ERG was expressed in about 5 morphologically benign glands across 418 cores and was uniformly expressed by all cancerous glands in 70 of 71 cores (99 %). Staining in precursor and atypical foci from our and other studies is discussed below. Importantly, other studies have also demonstrated the feasibility of ERG evaluation by IHC on needle biopsy cores [65, 66], confirming the ability of ETS gene fusions to be evaluated in the diagnostic setting.

As described above, ETS rearrangements are highly clonal in a given prostate cancer focus, and in our experience, the vast majority of foci show uniform strong, nuclear ERG staining, particularly in cases which would require immunohistochemical workup [32, 65, 75, 141]. Although foci may show varying levels of staining [66], finding ERG positive and negative cancer glands in what is unequivocally the same cancer foci is extremely rare. Similarly ERG IHC also stains wild type

ERG in blood vessels, and with minimal experience the staining in prostate cancer and vessels can easily be distinguished, and in our experience, provide little diagnostic difficulty. These features make ERG IHC ideal for evaluation in limited samples where diagnostic difficulty often arises.

Ideally, a prostate cancer-specific biomarker would never be expressed in benign prostatic glands or benign mimickers. At present, diagnostic workup of challenging cases often includes IHC for AMACR as a prostate cancer-specific biomarker. AMACR, lacks 100 % cancer specificity, as it can be negative in ~20 % of unequivocal prostate cancer, is positive in a subset of benign mimics of prostate cancer (including adenosis and partial atrophy) and may show focal staining in up to 20 % of morphologically benign glands [142–145]. Additionally, variability in staining may result in strong AMACR expression in cancer, with weaker expression in benign prostate glands. Nevertheless, AMACR is diagnostically useful and can often be used to convert an atypical diagnosis to a diagnosis of carcinoma [145, 146].

In comparison to AMACR, ERG is at least an order of magnitude more specific for prostate cancer. We are not aware of high quality reports demonstrating *ERG* rearrangements by FISH in benign prostate tissue. Similarly, as described above, two studies on prostatectomy sections demonstrate at least 99.99 % specificity of ERG staining for prostate cancer, with other studies on more limiting samples rarely, if ever, showing ERG staining in benign glands [32, 64–67, 69–72, 74, 75, 77, 141, 147]. In our study of both challenging diagnostic biopsies and cores from all cases signed out over a 3-month period, we found 2 foci of ~5 benign glands staining for ERG across 397 cores. Similarly, Van Leender's et al. found that 5 of 87 needle biopsies contained benign glands with focal weak ERG staining; in all cases, these glands were adjacent to ERG staining carcinoma [65].

HGPIN, where architecturally benign acini or ducts are lined by cytotypically atypical cells, is the presumed precursor lesion of prostate cancer, and can be challenging to differentiate from prostatic carcinoma on needle biopsy. In particular, when the typically larger glands of HGPIN have adjacent smaller glands, architecturally suggestive of carcinoma, it can be impossible to differentiate tangential sectioning of HGPIN and HGPIN with adjacent carcinoma, even with IHC, AMACR, and basal cell markers. This leads to a diagnosis of HGPIN with adjacent atypical glands (PINATYP). Hence, an ideal biomarker would not be expressed in HGPIN and instead only expressed in frank carcinoma.

Importantly, AMACR is expressed in virtually all foci of HGPIN [145, 148]. By FISH, multiple studies have shown that *ERG* rearrangements are detectable in approximately 15 % of HGPIN lesions, invariably adjacent to *ERG* rearranged prostate carcinoma [91, 92, 102]. For example, Han et al. found that 11 of 15 (73 %) HGPIN foci adjacent to carcinoma had *ERG* rearrangements (all 11 adjacent to *ERG* rearranged carcinoma), while 0 of 10 HGPIN foci distant to carcinoma had *ERG* rearrangements (even though 8/10 carcinomas were *ERG* rearranged) [91]. Multiple studies using IHC have found that ERG positive HGPIN is also nearly invariably adjacent to cancer that is also positive for ERG staining [32, 64, 65, 70]. For example, van Leenders studies a cohort of prostate needle biopsies containing

cancer and found that ERG stained 11 of 21 (52 %) HGPIN foci, in all cases adjacent to ERG positive cancer (10 of 11 foci had residual cancer in the core for comparison). Together these studies demonstrate that unlike AMACR, which stains nearly all foci of HGPIN, ERG stains only a subset of HGPIN, which are nearly always located immediately adjacent to ERG staining cancer.

Clinical Utility of ETS Gene Fusions as a Tissue Biomarker of Prostate Cancer

ERG in the Workup of Atypical Foci on Diagnostic Biopsy

The above data provide the context to evaluate the potential of ETS gene fusions (predominantly *ERG*) as a tissue-based biomarker. Here we will discuss three of the most promising uses for ERG in tissue-based diagnosis, including work up of atypical foci, risk stratification of HGPIN, and the diagnosis of small cell carcinoma of the prostate. As described above, ERG expression is extremely prostate cancer specific, with exceptionally rare staining in benign glands, which are nearly always adjacent to ERG positive carcinoma. Similarly, ERG is only expressed in a subset of HGPIN, which is also nearly always adjacent to ERG positive carcinoma. Hence, the simplest explanation in a needle biopsy specimen for ERG expression in a focus that appears morphologically benign, or a focus of HGPIN, is that adjacent, unsampled ERG expressing cancer is highly likely to be present. A primary challenge for pathologists is the evaluation of limited foci that do not have unequivocal quantitative or qualitative features of prostate cancer, often leading to an atypical diagnosis, or atypical small acinar proliferation (ASAP). Importantly, these cases are often worked up using immunohistochemistry for AMACR and basal cell markers, which may be able to rule out a diagnosis of cancer (if basal cell markers are present), or support a diagnosis of cancer (if AMACR is positive and/or basal cell markers are negative), although the diagnosis is still primarily morphological [148].

He et al. identified ERG staining in 16 of 103 (15.5 %) atypical biopsies [147]. Similarly, in our study of needle biopsies, ERG staining was noted in 3 of 28 (11 %) cores with atypical foci (all three diagnosed as ASAP) [75]. However, all cores in both studies were diagnosed prior to evaluation of ERG staining. Hence, these studies do not directly address the ability of ERG staining to add to current diagnostic IHC in the workup of challenging cases. Additionally, pathologist's threshold for calling lesions atypical or prostate cancer (both with and without diagnostic IHC) complicates assessment of the usefulness of a novel biomarker. He et al. found no significant difference in rates of cancer in follow-up biopsies from patients with ERG staining positive or negative atypical biopsies [147]. However, results from numerous FISH and IHC based studies suggest that ERG positive atypical or HGPIN foci only indicate risk for developing/harboring unsampled cancer immediately adjacent to that ERG positive focus, and hence prospective studies with targeted

re-biopsy may be required to truly assess the utility of ERG staining in diagnostically challenging samples. Just recently, Shah et al. reported that ERG staining was able to resolve atypical diagnostic cores to prostate cancer in 28 % of cases whose diagnoses would otherwise remain atypical based on AMACR and basal markers [149]. Remarkably, 10 of 12 cases converted to a cancer diagnosis based on positive ERG staining underwent repeat biopsy, and all 10 had cancer on the repeat specimen.

At present, we utilize ERG on selected challenging needle biopsy specimens, either concurrent with or after staining for AMACR and basal markers, where positive ERG staining may convert an atypical diagnosis to carcinoma, or where ERG staining would provide more confidence in the diagnosis of carcinoma. In our experience (S.A.T.), as ERG does not stain architectural mimickers of cancer, such as adenosis or partial atrophy [75, 141], ERG expression in an atypical focus without basal cells where HGPIN can be excluded indicates cancer, regardless of an AMACR staining. Similarly, although ERG expression in both the large glands and small adjacent glands of PINATYP does not support an unequivocal diagnosis of prostate cancer, ERG expression exclusively in the smaller glands is highly suggestive of carcinoma. ERG IHC in a representative focus of carcinoma and on a diagnostically challenging core is presented in Fig. 5.6.

ERG for the Risk Stratification of Isolated High Grade Prostatic Intraepithelial Neoplasia

Presently, the risk of cancer on rebiopsy after a diagnosis of isolated High Grade Prostatic Intraepithelial Neoplasia (HGPIN) is ~25 %, and clinicopathological parameters are unable to reliably identify men with increased risk of cancer on rebiopsy [148]. Based on the association of *ERG* (or *TMPRSS2*) rearranged or expressing HGPIN and similarly rearranged or expressing prostate cancer, we feel that ERG positive HGPIN indicates unsampled adjacent prostate cancer, or HGPIN that will inevitably progress to invasive disease. However, only a single study has directly addressed this question, with Gao et al. evaluating a Chinese cohort of 162 patients diagnosed with HGPIN on biopsy by FISH and IHC for *ERG* rearrangements and expression, respectively [150]. Interpretation of this study is complicated by the use of non-standard FISH evaluation (counting signals in at least 400 cells, regardless of HGPIN focus size), which led to cutoffs of < or > 1.6 % of cells with *ERG* aberrations being called *ERG* rearrangement positive or negative (in our experience almost all HGPIN foci have less than 400 evaluable nuclei, and counting only HGPIN cells, >75 % of cells harbor rearrangements in positive foci and <5 % harbor rearrangements in negative foci). The authors did however also perform IHC for ERG, which is simpler to evaluate, and reported high concordance with FISH. These limitations notwithstanding, the authors reported that on follow-up biopsy, 56 of 59 patients (95 %) with *ERG* rearranged HGPIN were diagnosed with carcinoma (all in the same zone as the HGPIN), compared to only 5 of 103 (5 %) patients with *ERG* wild-type HGPIN.

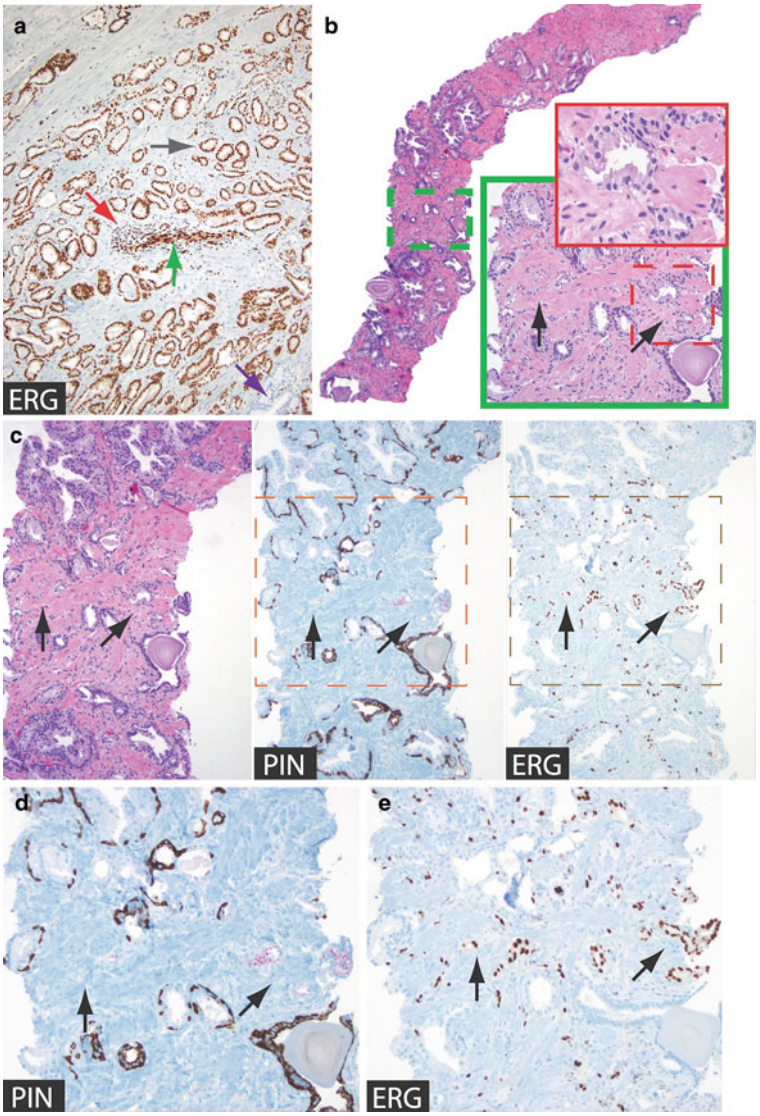

Fig. 5.6 *ERG immunohistochemistry (IHC) in prostate cancer*. (**a**) Typical ERG staining in a focus of prostate cancer using the EPR3863 antibody. ERG shows strong, diffuse nuclear staining in prostate cancer foci (*gray arrow*) harboring *ERG* rearrangements. Staining is not present in adjacent benign glands (*purple arrow*). ERG antibodies used for IHC also detect wild-type ERG (and may cross react with the related ETS protein FLI1), which results in diffuse strong nuclear staining in blood vessels (*green arrow*) with variable staining in tissue lymphocytes (*red arrow*). Original magnification 10×. (**b**–**e**) Utility of ERG IHC in the diagnostic work-up of challenging cases. (**b**) A 12 core needle biopsy had a single core with a small focus of architecturally and cytologically suspicious glands (*black arrows*). Original magnification 4×, 20× (*green box*), and 40× (*red box*). (**c**) The core was assessed by IHC for basal cell markers (p63 and high molecular weight cytokeratin, brown chromogen) and AMACR (red chromogen) in a cocktail (PIN, *middle* panel), and ERG (brown chromogen, *right panel*). Original magnification 10×. (**d**–**e**). Higher power views of the areas indicated in (**c**) (*middle* and *right panel*). The suspicious glands are positive for AMACR and ERG and negative with basal cell markers, consistent with prostatic adenocarcinoma. Original magnification 20×

These findings will need to be confirmed in well-designed studies, ideally those where all patients with HGPIN receive standard follow-up biopsy(s), including targeted biopsies toward zones with HGPIN (hypothesizing that ERG positive HGPIN will be associated with increased cancer risk only in those zones). Thus, until such studies have been reported, we do not routinely perform ERG immunostaining on HGPIN, however, if after workup of an atypical focus we make a diagnosis of ERG positive HGPIN, we will include a comment that multiple published studies indicate that ERG positive HGPIN is highly likely to be adjacent to ERG positive carcinoma and rebiopsy is recommended (S.A.T.).

Clinical Utility of ETS Gene Fusions as a Prostate Specific Cancer Marker: Small Cell Carcinoma

An important feature of ETS gene fusions is that they are not only highly cancer specific in the context of the prostate, they are also highly specific for prostate cancer compared to other cancers. For example, AMACR, is expressed in a number of other cancers [151], including other genitourinary cancers (such as papillary type renal cell carcinoma). On the other hand, ETS gene fusions are highly specific to prostate cancer, as the other malignancies known to harbor ETS gene fusions (Ewing's sarcoma and acute myeloid leukemia) are not in the differential diagnosis. For example, Scheble et al. assessed 2,942 samples (representing 54 different tumor types) for *ERG* rearrangement status by FISH and SNP copy number data from 3,131 cancer specimens (representing 26 *tumor* types) [152]. They found that none of the 54 different tumor types assessed by FISH harbored an ERG rearrangement, and in the 26 tumor types assessed for copy number alterations, the deletion site between *TMPRSS2* and *ERG* (on chromosome 21) was only identified in prostate cancer. Similarly, two IHC-based studies have found that ERG overexpression is exceptionally rare across other tumors (with the exception of Ewing's tumors, as well as vascular tumors [such as angiosarcomas], where ERG is a valuable marker) [76, 153]. For example, Minner et al. evaluated over 11,000 tumors and 72 different normal tissue types for ERG expression by IHC. In addition to prostate tumors and vascular tumors, they found moderate to strong ERG expression only in a substantial fraction of thymomos (6 %) and weak to moderate ERG staining in rare subsets of other tumors. No ERG expression was present in the remaining 8,886 samples from 132 other tumor types and subtypes, and only normal vascular tissue and lymphocytes expressed ERG.

Several groups have shown that this prostate cancer specificity can be utilized in the evaluation of cancer of unknown primary where a poorly differentiated prostate cancer or small cell carcinoma of prostatic origin is in the differential. Importantly, in these situations, PSA and other lineage markers, such as NKX3.1, are often negative by IHC (as the tumor no longer has active androgen signaling) and thus immunohistochemistry often cannot be used to determine prostatic origin. Similarly, ERG by IHC has little utility in this scenario, as ERG expression is dependent on

androgen signaling in all known *ERG* gene fusions, and in our experience is never positive in the absence of PSA expression. However, multiple groups have now shown that many poorly differentiated, androgen signaling negative tumors continue to harbor *ERG* rearrangements at the genomic level, presumably indicating that the androgen signaling negative tumor arose from an androgen signaling positive tumor. For example, Hermans et al. identified *TMPRSS2:ERG* transcript overexpression in 5 of 5 (100 %) of androgen signaling positive prostate cancer xenografts with *ERG* rearrangements, but no transcript expression in 3 androgen signaling negative prostate cancer xenografts and two AR negative clinical prostate cancer specimens all with ERG rearrangements [35].

Multiple groups have now directly assessed the diagnostic utility of *ERG* rearrangement detection in establishing the origin of poorly differentiated or small cell carcinoma where prostatic primary is in the differential [154–158]. As examples, Scheble et al. identified *ERG* rearrangements in 13 of 15 (86 %) prostatic small cell carcinomas, but 0 of 22 pulmonary small cell carcinomas, and *ERG* rearrangement status was the best marker to differentiate the two tumor types [157]. Similarly, Lotan et al. demonstrated that 10 of 22 (45 %) prostatic small cell carcinomas harbored *ERG* rearrangements with concordant *ERG* rearrangement in 5 of 6 (83 %) cases with a conventional adenocarcinoma component, while *ERG* rearrangements were absent in bladder ($n = 12$) and pulmonary ($n = 13$) small cell carcinomas [157]. Consistent with loss or reduced androgen signaling in prostatic small cell carcinomas, ERG (23 %), AR (27 %), and NKX3.1 (18 %) expression by IHC were infrequent in prostatic small cell carcinomas, demonstrating the superiority of FISH for *ERG* rearrangement detection in this situation. Together, these studies convincingly demonstrate that the presence of an *ERG* rearrangement is highly specific for cancer of prostatic origin, including small cell carcinoma and evaluation of by FISH should be considered in the correct clinical scenario.

Clinical Utility of ETS Gene Fusions in the Clonal Evaluation of Prostate Cancer

Lastly, as *ERG* rearrangements are highly clonal when present in a focus (and don't change during cancer progression), they may have specific utility in clinical scenarios where clonal evaluation is needed. For example, in needle biopsy cores with small discontinuous foci of cancer at each end of the core, Karram et al. have recommended reporting the core as 100 % involved using supporting data from prostatectomies [159]. Similarly, discordant ERG staining in cancer foci on different needle biopsy cores would indicate that the patient has multifocal cancer. Whether such findings would improve the prediction of tumor size will need to be addressed. As FISH can stratify ETS + tumors by rearrangement mechanism (deletion or insertion), it may have increased utility compared to IHC concerning issues of clonality or lesion tracking.

Clinical Utility of Urine TMPRSS2:ERG *as an Early Detection Biomarker*

Given their extremely high cancer specificity, ETS gene fusions have also been evaluated as biomarkers for early detection of prostate cancer. As ETS gene fusion protein products are not secreted, and the most common *TMPRSS2:ERG* gene fusion does not encode a chimeric, antibody-based detection in serum (as for PSA) is not feasible. However, multiple groups have demonstrated that *TMPRSS2:ERG* transcripts are detectable in post-digital rectal exam urine collected from men with (or at risk for having) prostate cancer [160–170].

Due to concerns of sensitivity limitations of *TMPRSS2:ERG*, multiple groups have investigated multiplexing urine *TMPRSS2:ERG* with other prostate cancer markers for predicting the presence of cancer. For example, Hermans et al. then demonstrated the potential utility of combining urine *TMPRSS2:ERG* with urine assessment of *PCA3* [162], a gene that encodes a noncoding transcript overexpressed in approximately 90 % of prostate cancers [171]. Similarly, Laxman et al. demonstrated the utility of combining urine *TMPRSS2:ERG* with urine *PCA3*, *GOLPH2* and *SPINK1* for predicting the presence of cancer on biopsy [164]. Additional studies, including those by Salami et al. and Cao et al. further demonstrated the utility of combining urine *TMPRSS2:ERG* with other markers, including *PCA3* [165, 167].

Importantly, the majority of studies have been performed using research grade assays for *TMPRSS2:ERG* (often RT-PCR-based assays) and are reporting *TMPRSS2:ERG* transcript as present or absent. Although *TMPRSS2:ERG* expression in an individual cancer focus is either uniformly present or absent (present in about 50 %), it does not follow that 50 % of men with prostate cancer will have *TMPRSS2:ERG* in their urine. First, prostate cancer is often multifocal as described above, suggesting that more than 50 % of men with prostate cancer have at least one focus that is *TMPRSS2:ERG* positive. Secondly, although "present" or "absent" by FISH or IHC in tissue specimens, the amount of urine detectable transcript should not be the same from a 0.1 cm^3 *TMPRSS2:ERG* expressing focus and a 3.0 cm^3 *TMPRSS2:ERG* (given comparable rates of shedding into the urine).

In 2011, we reported on the development and validation of a clinical grade, transcription-mediated amplification assay for the quantification of *TMPRSS2:ERG* in post-digital rectal exam (DRE), whole urine [168]. We applied the assay to prospectively collected, whole urine from 1,312 men at multiple centers presenting for biopsy or prostatectomy. Importantly, we found that urine *TMPRSS2:ERG* was associated with indicators of clinically significant cancer at biopsy and prostatectomy, including tumor size, high Gleason score at prostatectomy, and upgrading of Gleason grade at prostatectomy. *TMPRSS2:ERG*, in combination with urine *PCA3*, improved the performance of the multivariate Prostate Cancer Prevention Trial risk calculator (PCPTrc) in predicting cancer on biopsy [168]. Similarly, Cornu et al. evaluated urine *TMPRSS2:ERG* by the TMA assay in 291 patients undergoing biopsy and found that *TMPRSS2:ERG* score, *PCA3* score, and PSA density were all

correlated with presence of cancer on biopsy in a multivariate model, with *TMPRSS2:ERG* score and *PCA3* showing nearly equivalent AUCs (0.67 and 0.66, respectively) [170].

Recently we performed a pilot study to investigate the correlation between tissue expression of *TMPRSS2:ERG* (by IHC for ERG) and urine *TMPRSS2:ERG* [141]. From 41 patients undergoing prostatectomy, we assessed urine *TMPRSS2:ERG* and *PCA3* from pre-biopsy post-DRE urine and mapped all prostate cancer foci and stained each focus for ERG expression. Importantly, we found that 37 of 41 patients had multifocal tumors, with 32 of 41 (78 %) patients having at least one ERG$^+$ focus (vs. 49 % of all individual foci being ERG$^+$). Our evaluation of ERG expression in tissue was consistent with the extreme cancer specificity described above, as we found that across 169 quartered prostatectomy sections, only 35 total benign glands across 9 cases were ERG$^+$, with only a single focus greater than 0.4 cm from ERG$^+$ cancer. Given the number of benign glands per section [64], we found that ERG staining is greater than 99.99 % specific for prostate cancer [140]. Importantly, we found that urine *TMPRSS2:ERG* was strongly correlated to the total volume of ERG$^+$ tumor (calculating by summing the largest dimension of all ERG$^+$ foci) and the total number of ERG$^+$ foci ($r_s = 0.68$ and 0.67, $P < 0.0001$ each). Interestingly, urine *PCA3* was less correlated with the total volume of tumor ($r_s = 0.26$), suggesting that *TMPRSS2:ERG* is a more specific cancer biomarker. Taken together, these data support the cancer specificity of urine *TMPRSS2:ERG*, which combined with *PCA3*, improves the ability to predict the presence of prostate cancer in men with "elevated" serum PSA. This suggests potential utility of urine *TMPRSS2:ERG*, the most cancer specific biomarker yet reported for prostate cancer, in the early detection of prostate cancer regardless of serum PSA levels.

Clinical Utility of ETS Status for Predicting "Outcome" in Prostate Cancer

One of the major challenges in the management of prostate cancer is identifying patients who have indolent cancer and need less (or no) treatment vs. those who have aggressive cancer and need aggressive therapy or multimodality therapy. Although many studies have attempted to correlate ETS status with outcome in prostate cancer, these studies must be interpreted in the context of how the cancer was diagnosed and managed, and how "outcome" was determined. Essentially, there are 5 types of "outcome" studies (1) incidentally detected (non-PSA screened) cancer by TURP, no treatment, (2) incidentally detected (non-PSA screened) cancer by TURP, radical prostatectomy, (3) biopsy detected (PSA-screened), no treatment, (4) biopsy detected (PSA-screened), radical prostatectomy, and, (5) incidentally or biopsy detected, radiation therapy. Below, we will describe the results from studies using this stratification paradigm. Importantly, we are not aware of studies addressing ETS status in non-PSA screened, radical prostatectomy treated (clinical scenario 2 above), and as this cohort likely does not exist currently in the USA given

the rate of PSA screening, we will not discuss it further. Similarly, we are not aware of published studies assessing ETS status in patients treated by radiation therapy (clinical scenario 5). Importantly, the difficulty in evaluating outcome and ETS status has been reviewed recently [2], and comprehensively studied in a recent meta-analysis [49].

ETS Status in Incidentally Detected Untreated Cancer

Multiple studies have investigated associations between ETS status and outcome in this context. Demichelis et al. initially reported that *ERG* rearrangements were associated with a threefold increase in prostate cancer death compared to non-*ERG* rearranged cancers in a cohort of 111 men diagnosed by TURP and managed conservatively [81]. Later, a larger cohort of patients was studied by this group, who reported that 41 of 46 patients with an *ERG* rearrangement eventually died from prostate cancer [172]. Similarly, Attard et al. and Reid et al. assessed *ERG* (and *ETV1*) rearrangements in a cohort of 308 men diagnosed by TURP and managed conservatively and found that the presence of an *ERG* or *ETV1* rearrangement was associated with increased risk of death from prostate cancer; however, this was not independent of Gleason score [51, 80]. Importantly, these studies demonstrated low rates of *ERG* gene rearrangements, as described above under the discussion of prevalence. Hence, it is unclear if the aggressive cancers harboring ETS gene fusions in these cohorts represent peripheral zone tumors that have grown into the transition zone (which are then detected by TURP), or if these are aggressive tumors arising in the transition zone, which typically has less aggressive tumors than the peripheral zone.

ETS Status in PSA Screened, Radical Prostatectomy Treated Cancer

Numerous studies have now attempted to associate ETS gene fusions with outcome after radical prostatectomy (see reference [49] for review). This is in large part due to tissue availability and the ability to make tissue microarrays from prostatectomy samples. Importantly, the vast majority of these studies have sampled a single focus (the index nodule), which based on size or Gleason score is presumed to be the focus driving the biological behavior after prostatectomy. Similarly, most studies use PSA recurrence as the outcome measure, which is not a reliable indicator of eventual death from prostate cancer [173–175].

With these caveats, analyses from large, well-annotated cohorts do not show associations of ETS gene fusions with outcome in PSA screened, radical prostatectomies, as recently reviewed by Petterssen et al. [49]. For example, Minner et al. recently investigated ETS status in a cohort of 2,805 men undergoing prostatectomy. ERG expression by IHC was found in 52 % of cancers, with 95 %

concordance between ERG expression and *ERG* gene rearrangement detected by FISH in the subset of cases examined [70]. The authors found no association between ERG expression and PSA recurrence. Interestingly, in their meta-analysis, Petterssen et al. reported an overall association of ETS gene fusions with advanced stage (extra-prostatic extension) at prostatectomy, but not PSA recurrence [49]. They suggest that ETS gene fusions may be associated with localized tumor growth rather than metastatic spread; thus in men treated by radical prostatectomy (who are most often treated while the tumor is confined to the prostate), ETS fusions would not predict outcome. Additionally, although *TMPRSS2:ERG* fusions through deletion have been associated with more aggressive behavior than fusions through insertion [78], and in particular duplication of the ETS gene fusion, this association was not found by meta-analysis [49].

ETS Status in PSA Screened, Untreated Cancer

Given the association of ETS gene rearrangements and death from prostate cancer in incidentally detected, conservatively managed prostate cancer, it seems logical that ETS rearrangements may be associated with aggressive behavior in PSA screened conservatively managed prostate cancers. Importantly, in the USA, almost all patients undergoing conservative management have low volume, Gleason 6 cancers, and undergo definitive therapy if Gleason 7 cancer, or substantial tumor volume, is detected on follow-up biopsy. Additionally, a large percentage of men undergo definitive therapy in the absence of adverse pathology, given the need for continued rectal exams, serum PSA tests and prostate biopsies, and the anxiety of having cancer. Hence, it will likely not be possible to truly study the natural history of PSA-detected, conservatively managed ETS^{+} vs. ETS^{-} prostate cancer. Nevertheless, studies are needed to evaluate the ability of ETS status at baseline to predict active surveillance failure and upgrading or upstaging at prostatectomy in these patients.

ETS Gene Fusion Status as the Basis for Comprehensive Molecular Subtyping of Prostate Cancer

Additionally, given the advancements made in subtyping prostate cancer as described above, studies are now beginning to address the utility of further molecular subtypes in studies similar to those just described. For example, multiple studies have now addressed ETS/*PTEN* status for predicting outcome in both conservatively managed (non-PSA screened) and radical prostatectomy treated (PSA-screened cohorts) [51, 127, 128]. For example, Reid et al. found that in conservatively managed patients, those with wild-type ETS and *PTEN* (ETS^{-}/$PTEN^{wt}$) had the best outcome [51]. In prostatectomy patients, Yoshimoto et al. also reported that

ETS$^-$/*PTEN*^{wt} patients also had the best outcome [127], while Krohn et al. found that *PTEN* deletion was associated with poor outcome regardless of ETS status in their large cohort of RRP patients [128]. However, using the same cohort, Muller et al. reported that loss of pSer2448-mTOR expression was associated with poor outcome only in ERG$^+$ prostate cancer and identified a subset of ERG$^+$/PTEN$^-$ with very poor outcome [176]. It is likely that as comprehensive subtyping becomes feasible, either through a limited series of markers (such as ETS fusions, *PTEN*, *TP53*, *RB1*, RAS, and RAF family members, *CHD1* and *SPOP*) further associations with outcome in different cohorts will be addressable.

ETS Gene Fusion Status for Subtyping the Medical Management of Prostate Cancer

Therapeutic options for patients with low-intermediate risk-localized prostate cancer include active surveillance, radical prostatectomy, and radiation therapy. Prostatectomy and radiation may be used in combination (with or without anti-androgen treatment) in patients with high risk disease or locally advanced disease. Anti-androgens are typically the frontline treatment for metastatic prostate cancer (see below), and upon disease progression, chemotherapy (typically docetaxel) has been the mainstay of treatment. Given that the vast majority of patients with prostate cancer in need of medical therapy were treated identically, our lack of understanding of the mechanisms driving disease progression and recurrence, and few validated therapeutic targets (other than the androgen receptor), there has been little impetus and progress in identifying molecular subtypes of prostate cancer and targeted therapies for these subgroups. Unlike many common epithelial cancers, such as breast and lung, where we are actively identifying groups as small as 1–5 % for targeting (such as identifying patients harboring *ALK*, *ROS*, or *RET* rearrangements in lung cancer for treatment with crizotinib [177–181]), molecular subtyping currently plays no role in the management of prostate cancer. However, the enormous gains in our understanding of the biology of prostate cancer have led directly to several novel agents being recently approved to treat metastatic prostate cancer, with more promising therapeutics in (or recently completed) late stage trials, meaning that we will soon have multiple treatment options available for patients. Hence, we feel that molecular subtyping and treatment of prostate cancer is at inflection point and we expect the classification and treatment of prostate cancer to be transformed in the next decade.

As described above, ETS gene fusion status serves as a robust marker for the molecular subtyping of prostate cancer, and as described below, may also play a key role in the treatment of prostate cancer. For example, ETS gene fusion status may play a driving role in determining a patient's response to therapy either indirectly, such as through the effects of anti-androgens, or directly, through novel agents that target the function of ETS gene fusions directly. An overview of potential strategies to target ETS gene fusions (predominantly ERG) is shown in Fig. 5.7.

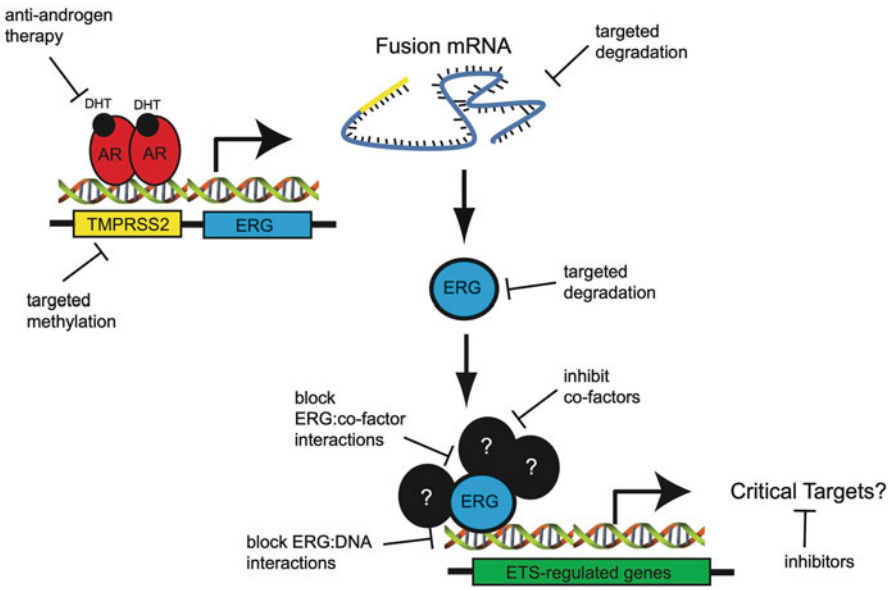

Fig. 5.7 *Schematic representation of potential strategies to inhibit the TMPRSS2:ERG transcription factor signaling axis*. Potential therapies include blocking androgen receptor (AR)-mediated transcription (AR antagonists, targets DNA silencing), anti-sense RNA approaches, induction of protein degradation, cofactor inhibitors, molecules that block either ERG:cofactor and/or ERG:DNA interactions as well as inhibitors of critical downstream components of the signaling axis

In addition, given the robustness of subtyping based on ETS gene fusion status, specific molecular subtypes of prostate cancer (i.e., ETS^{-}/*SPINK1*$^{+}$) may respond preferentially to agents, even if they do not directly target the lesions used in subtyping. An analogous situation is the identification PARP inhibitor activity in early clinical trials of *BRCA1* mutant breast cancers, which are enriched in the subgroup of *ER*$^{-}$/*PR*$^{-}$/*HER2*$^{-}$ (triple negative) breast cancers. Here, we will describe in detail the potential utility of ETS gene fusions in current as well as future therapies.

ETS Gene Fusions in the Response to Anti-Androgen Therapy

While 80–90 % of men with high risk or metastatic prostate cancer receive and generally respond well to androgen deprivation therapy, nearly all men eventually relapse with castration-resistant prostate cancer (CRPC). Although this has often been called “androgen insensitive” disease in the past, this term is incorrect, as androgen signaling is commonly restored in these cancers, which simultaneously restores the ETS gene fusion axis [112] and results in continued expression of ETS gene fusions [61, 182]. For example, in our recent integrative mutational profiling

study of CRPC, we found that 30 of 48 (63 %) CRPCs had point mutations or high level amplification of *AR*, demonstrating the selective pressure to restore androgen signaling upon anti-androgen therapy [61]. Similarly, 17 of the 21 (81 %) CRPCs in our cohort with androgen-driven ETS gene fusions still had outlier overexpression of the involved ETS gene (most commonly *ERG*), demonstrating continued activation of androgen signaling.

Based on similar observations of continued activation of the AR signaling axis in CRPC [183–185], several second- and third-generation AR antagonists have been developed, including abiraterone, MVD3100 (enzalutamide), and ARN-509. Given the role of androgen in driving almost all ETS gene fusions, a natural question is whether anti-androgen therapy works preferentially in patients harboring ETS gene fusions.

Recently, in a small pilot study, Lehmusvaara et al. demonstrated that the presence of *TMPRSS2:ERG* gene fusions sensitizes prostate cancer at the molecular level to anti-androgen therapy, as anti-androgen therapy downregulated nearly ninefold more genes in cases harboring *ERG* rearrangements compared to *ERG*$^{-}$ cases (601 vs. 69 genes), of which the main transcriptional program was related to the cell cycle [186]. Leinonen et al. evaluated *ERG*/*SPINK1* status (by FISH and IHC, respectively) in 178 patients treated by primarily anti-androgen therapy and found that 34 % of patients were *ERG*$^{+}$, which was not associated with progression free survival; however, high *SPINK1* expression was found in 11 % of cases and was associated with shorter progression free survival compared to *SPINK1* negative cases, independently of other clinicopathological variables [187]. Similarly, Boormans et al. evaluated *TMPRSS2:ERG* status by RT-PCR in 85 patients with nodal involvement at prostatectomy and found that 59 % of the 71 evaluable patients harbored *TMPRSS2:ERG* fusions, which did not predict median duration of response to endocrine therapy [188].

Studying 77 patients with CRPC being treated with abiraterone on phase I/II trials, Attard et al. found that 32 (41 %) of patients harbored an *ERG* rearrangement (either in the primary tumor or in CTCs), and the presence of an *ERG* rearrangement was associated with the magnitude of maximal PSA decline ($P = 0.007$), with *ERG* rearrangements in 12 of 15 patients (80 %) and 20 of 62 patients (32 %) with ≥90 % and <90 % PSA decline, respectively ($P = 0.001$) [113]. However, Danilla et al. evaluated *TMPRSS2:ERG* status by RT-PCR in CTCs from 41 patients being treated with abiraterone and found that a PSA decline ≥50 % was observed in 7 of 15 patients (47 %) with *TMPRSS2:ERG* fusions, and in 10 of 26 patients (38 %) without *TMPRSS2:ERG* fusions, which was not significantly different [189].

Given the additional second- and third-generation anti-androgens that will likely soon be in the clinical armamentarium, identifying biomarkers of sensitivity and resistance, particularly in real time, will likely become a pressing clinical need. Taken together, the present data suggest that the effects of ETS gene fusions in the response to anti-androgens need further evaluation. However, as ETS gene fusions are almost always driven by androgen, continued studies on their effect on the efficacy of anti-androgens in the neoadjuvant, pre- and post-chemotherapy CPRC are warranted.

Indirect Therapeutic Targeting of ETS Gene Fusions Through PARP1 and DNA-PKcs

Despite the clear pathogenic role of aberrant transcription factor activity in a wide array of diseases, these proteins are currently considered to be "undruggable" molecular targets [190]. Because transcription factors are dependent on the ability to assemble protein interaction networks at various genomic loci [191], perhaps the most promising long-term strategy is to develop inhibitors of transcription factor:cofactor interactions. To date, however, these interactions are currently intractable, and the quest to identify inhibitors of any protein:protein interactions is impeding a move to personalized medicine for transcription factor-driven malignancies.

Our group has recently addressed the potential indirect targeting of ETS gene fusions, by first establishing the ETS protein interactome in prostate cancer. Using immunoprecipitation–mass spectrometry experiments, we demonstrated that ETS proteins interact with two chemically tractable enzymes: the *catalytic subunit of the DNA-dependent protein kinase* (DNA-PKcs) as well as *poly(ADP)-ribose polymerase 1* (PARP1) [101]. Excitement over this discovery stemmed from papers published in 2005, in which two groups demonstrated that *BRCA1/2* mutant cancer cells, which are unable to effectively execute a DNA double strand repair process called homologous recombination, are highly sensitive to PARP inhibition [192, 193]. In fact, because of those two papers the concept of synthetic lethality—a combination of disruptive events in two or more proteins that leads to cell death—was revitalized as a paradigm in cancer research. In this case, the synthetic lethality is between a mutation in either *BRCA1* or *BRCA2* and induced by chemical disruption of a second protein, PARP1. The initial clinical trials using the PARP inhibitor olaparib, demonstrated early efficacy in a phase I clinical trial, with limited side effects [194]. However, the precise mechanism of how PARP inhibition leads to cell death in *BRCA1/2* mutant cancer cells remains unknown (reviewed in [195]). Given the complexity and diversity of cellular processes in which PARP enzymes participate, the original model of *BRCA1/2* mutant-mediated PARP susceptibility is most likely overly simplistic and ongoing research is focused on identifying additional mechanistic details of the induced-sensitivity.

We demonstrated that PARP1 is required for transcription, invasion, intravasation, and metastasis of ETS positive prostate cancer cell lines in vitro [100]. Mechanistically, PARP inhibitors potentiate the baseline DNA damage caused by ETS gene fusion overexpression leading to long-term loss of cell viability consistent with mechanisms of cell death caused by increased DNA damage. Consistent with these findings, we demonstrated that ETS positive prostate cancer cell line xenografts were as sensitive to olaparib as a naturally *BRCA1* mutant breast cancer cell line xenograft while no significant changes were observed in short-term (<72 h) assays. Importantly, the addition of temozolomide to PARP inhibitor regimens caused a significant reduction in ETS positive xenograft growth suggesting that

combination therapies may be the most beneficial for patients. Additionally, through inhibition with either NU7026, a first-generation DNA-PKcs inhibitor, or siRNA, we demonstrated that DNA-PKcs is essential for ETS-mediated transcription and invasion in prostate cancer cell line models. Importantly, given the initial success of PARP inhibitors in both *BRCA1/2*-deficent cancers as well as preclinical ETS positive prostate cancer models, several clinical trials have been initiated to address their clinical utility in prostate cancer.

While our initial studies have indicated that biomarker-driven selection of patients may help predict the initial response of patients to PARP inhibitors, the clinical impact of these findings will soon be determined. Likewise, given their prevalence and potential as a prostate cancer-specific target, investigation by our group and others on the direct targeting of ETS gene fusions is ongoing.

Conclusions

The discovery of ETS gene fusions demonstrated the existence of molecular subtypes in prostate cancer, mirroring our increasing molecular understanding in other cancers. Here we have reviewed the role of ETS genes in normal tissues and cancer and described the discovery of their involvement in recurrent gene fusions in prostate cancer. Importantly, study of the diversity of ETS gene fusions, genesis, and oncogenic roles in vitro and in vivo provides important mechanistic details into the origin and molecular drivers in prostate cancer. Similarly, identification of additional collaborating or exclusive lesions provides a rational basis for the comprehensive molecular subtyping of prostate cancer. Given their extreme cancer specificity, ETS gene fusions have enormous potential as biomarkers, and we have described the rapid progress in assessing their utility in early detection and tissue-based diagnosis. We have also reviewed the potential of ETS gene fusions and associations with "outcome" which require careful analysis of the ETS gene fusion detection method, the clinical cohort characteristics (how was the cancer detected and treated) and how "outcome" is assessed. Importantly, these factors are important not just for evaluating ETS fusions but all prostate cancer biomarkers. Lastly, given their extreme cancer specificity, we have reviewed the potential of ETS gene fusions as both indirect and direct targets of therapy. A summary of the areas of current and potential clinical utility for ETS gene fusions in prostate cancer is shown in Fig. 5.8. Together, although it has been less than a decade since the discovery of ETS gene fusions, the research community has made enormous gains in understanding the genesis and role of ETS fusions in prostate cancer, and ETS gene fusion are begging to be applied as biomarkers in the clinic and will likely form the basis of clinical subtyping in the future. Similarly, as the most prostate cancer-specific biomarker yet reported, the potential of therapeutic targeting of ETS gene fusions (either directly or indirectly) may add to the recent advances made in treating men with advanced prostate cancer.

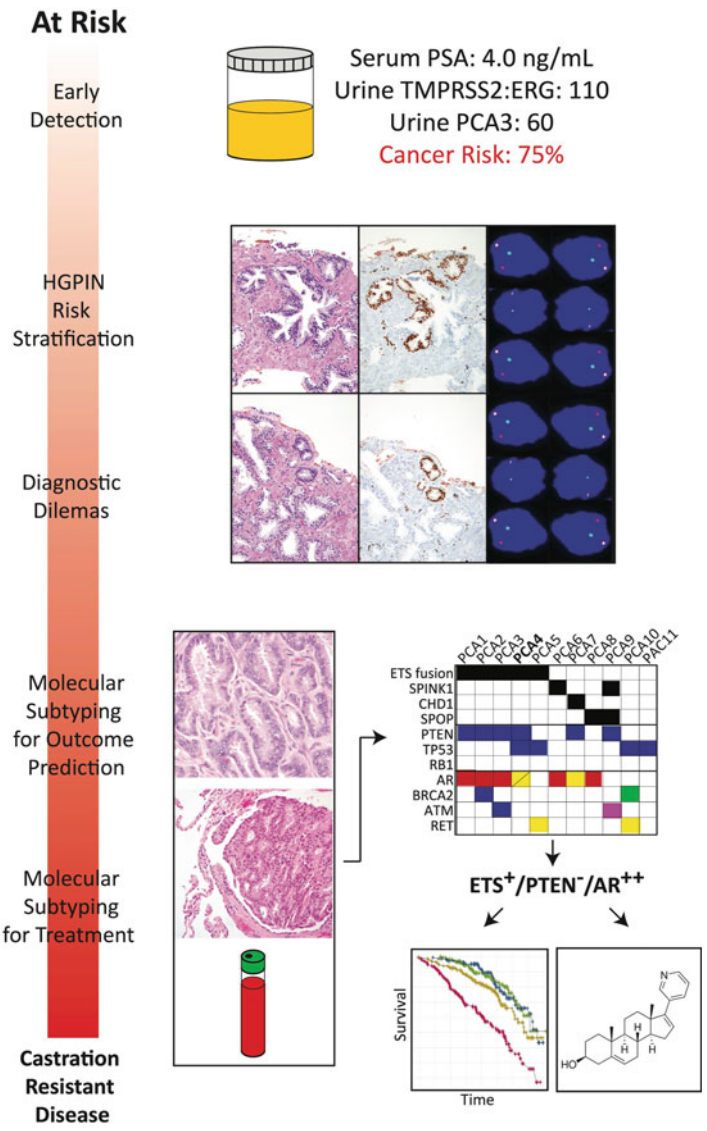

Fig. 5.8 *ETS gene fusions in the clinical management of prostate cancer*. Areas where ETS gene fusions can be used in the clinical management of prostate cancer, from men at risk, to those with castration resistant prostate cancer (CRPC) are shown. Quantification of *TMPRSS2:ERG* gene fusion transcripts in the urine of men at risk for having prostate cancer, combined with other markers and clinical information, can provide a more accurate prostate cancer risk estimation in the early diagnosis setting. Given the cancer specificity of ETS gene fusions for prostate cancer, evaluation of ERG status in high grade prostatic intraepithelial neoplasia (HPGIN), by immunohistochemistry (IHC) or fluorescence in situ (FISH), may be used to risk stratify men with isolated HGPIN on biopsy. Similarly, we utilize IHC for ERG in the workup of diagnostically challenging biopsies. We consider ETS status to be the basis for robust molecular subtyping of prostate cancer, which may have utility both for predicting outcome at the time of diagnosis or definitive treatment, as well as the optimum therapy for men with advanced disease. ETS status can be determined from diagnostic and resection specimens, as well as circulating tumor cells. Comprehensive assessment of ETS status, in combination with other markers identified through our increasing understanding of disease biology, will likely allow for tailored risk prediction and individualized therapy

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Part III
Cell Signaling

Chapter 6
Signaling Mechanisms of Vav3, a Guanine Nucleotide Exchange Factor and Androgen Receptor Coactivator, in Physiology and Prostate Cancer Progression

Leah S. Lyons and Kerry L. Burnstein

Abstract The Rho GTPase guanine nucleotide exchange factor (GEF) Vav3 is the third member of the Vav family of GEFS and is activated by tyrosine phosphorylation. Through stimulation of Rho GTPase activity, Vav3 promotes cell migration, invasion, and other cellular processes. Work from our laboratory first established that Vav3 is upregulated in models of castration-resistant prostate cancer progression and enhances androgen receptor as well as androgen receptor splice variant activity. Recent analysis of clinical specimens supports Vav3 as a potential biomarker of aggressive prostate cancer. Consistent with a role in promoting castration-resistant disease, Vav3 is a versatile enhancer of androgen receptor by both ligand-dependent and ligand-independent mechanisms and as such impacts established pathways of androgen receptor reactivation in advanced prostate cancer. Distinct Vav3 domains and mechanisms participate in ligand-dependent and -independent androgen receptor coactivation. To provide a physiologic context, we review Vav3 actions elucidated by gene knockout studies. This chapter describes the pervasive role of Vav3 in progression of prostate cancer to castration resistance. We discuss the mechanisms by which prostate cancer cells exploit Vav3 signaling to promote androgen receptor activity under different hormonal milieus, which are relevant to clinical prostate cancer. Lastly, we review the data on the emerging role for Vav3 in other cancers ranging from leukemias to gliomas.

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Introduction

While major strides have been made toward understanding the molecular changes that accompany the development of castration-resistant prostate cancer, the critical factors and pathways that drive this process are still being defined. Early microarray analyses were conducted to compare differences between androgen-dependent LNCaP cells and their castration-resistant derivative, LNCaP R1 cell line (developed by Drs. Kokontis and Liao from the University of Chicago) by long-term culture of LNCaP cells in androgen-depleted media [1]. These experiments revealed that the expression of the *VAV3* gene is robustly upregulated in the castration-resistant LNCaP R1 line as compared to LNCaP cells [2]. Indeed, Vav3 exhibited the highest degree of upregulation in LNCaP R1 cells compared to all other genes in this screen. Vav3 gene expression is now known to be elevated in several models of castration-resistant disease progression [3–8]. As the third and newest member of the Vav family of proteins, Vav3, like the other Vav family members, serves as a guanine nucleotide exchange factor (GEF), for small Rho GTPases [9]. While the closely related founding member of the Vav subfamily of diffuse B cell lymphoma (DBL) GEFs is considered a proto-oncogene because a truncated version of Vav1 (originating from a cloning artifact) is transforming [10], Vav3 was recognized originally only as a Rho GTPase GEF. Much work has revealed a broad spectrum of physiologic actions of Vav3 and unexpectedly that Vav3 plays a key role in prostate cancer progression by serving as a versatile coactivator of the androgen receptor (AR).

Vav Domain Composition and Regulation of GEF Activity

Vav3 belongs to a small subfamily of DBL GEF proteins that includes the closely related founding member, Vav1 and the Vav2 proteins [9]. Tissue distribution of Vav proteins varies among the family members. Vav1 is expressed primarily in hematopoietic tissues, whereas Vav2 is expressed ubiquitously and Vav3 has a broad expression profile, which includes prostate, heart, brain, bone, kidney, pancreas, placenta, and breast [11, 12] and data deposited in public databases (http://genecards.weizmann.ac.il/genenote/; http://www.ncbi.nlm.nih.gov/unigene/; http://biogps.org/). The Vav family of proteins is structurally complex, consisting of multiple functional domains. These domains include (sequentially from the amino terminus): a calponin homology (CH) domain, an acidic domain (AD), a catalytic DBL homology domain (DH), which confers GEF function, followed by a pleckstrin homology (PH) domain, a cysteine-rich domain (also termed a zinc finger domain), and two src homology 3 (SH3) domains flanking a single SH2 domain (Fig. 6.1). As is true for all DBL family GEFs, the DH domain interacts directly with Rho GTPases and the DH and PH domains are arranged in tandem [9, 13]. For most DBL family GEFS, the PH domain modulates GEF activity through

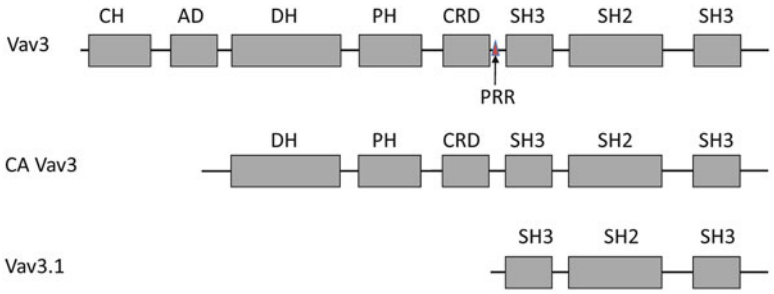

Fig. 6.1 Schematic representation of Vav3, constitutively active Vav3 (CA Vav3) and Vav3.1 domain organization. Domains are: *CH* calponin homology, *AD* acidic domain, *DH* DBL homology (GEF) domain, *PH* pleckstrin homology domain, *CRD* cysteine-rich domain, *SH3* src homology three domain, *SH2* src homology two domain. PRR represents the proline-rich region

interactions with membrane phosphoinositides [14, 15]. However, due to slight differences in three-dimensional structure, the Vav3 PH domain does not modulate Vav3 GEF activity [16, 17]. The Vav SH3 domains bind proline-rich regions, and the intervening SH2 domain is the site of interaction with receptor tyrosine kinase receptors responsible for Vav protein phosphorylation and activation of GEF function [9]. Residing just forward of the SH3–SH2–SH3 C-terminal cassette is a proline-rich region that may facilitate an intramolecular interaction with the C-terminal region [18] (and our unpublished observations). Additionally the Vav SH3–SH2–SH3 cassette participates in protein–protein interactions that are important for scaffolding and adaptor functions [18–20].

The mechanism of GEF function occurs through interaction of the Vav DH domain with Rho GTPases, resulting in stabilization of the nucleotide-free state of Rho proteins [13]. Since intracellular concentrations of GTP far exceed those of GDP, this process facilitates GTP binding to the Rho proteins [9, 13, 21]. Vav3 serves as a GEF for RhoA, RhoG, and Rac1 [22, 23] thereby activating these proteins and promoting signaling through downstream effectors to mediate diverse cellular responses including actin cytoskeleton reorganization, gene expression, and cell cycle regulation [24].

The GEF activity of Vav proteins is stimulated by phosphorylation of critical tyrosine residues on the Vav amino-terminal regions through the action of multiple receptor and non-receptor tyrosine kinases. More specifically, Vav proteins are recruited via their SH2 domains to phosphotyrosine residues on interacting proteins, including activated growth factor receptors, and are tyrosine phosphorylated [9, 23, 25]. Based on crystallography and other structural studies, these phosphorylation events initiate repositioning of a Vav amino-terminal autoinhibitory loop, which in the inactive protein functions to suppress GEF activity by physically blocking access of Rho proteins to the DH domain [9, 17, 26] (Fig. 6.2). Phosphorylation results in allosteric changes that relieve autoinhibition thereby activating GEF function [9, 17, 27]. In particular, tyrosine 173 of Vav3 is a critical residue in this process [9, 27]. Consistent with an autoinhibitory role of the

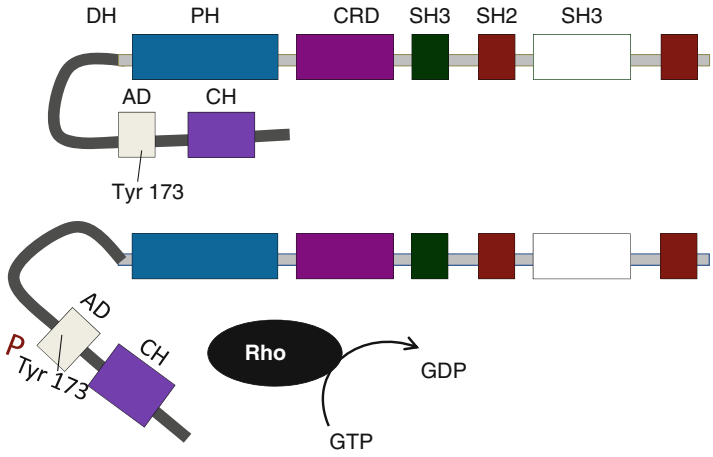

Fig. 6.2 Schematic showing inactive (*top panel*) and active (*bottom panel*) conformations of Vav3. Movement of the N-terminal regions to allow RhoGTPase access to the DH domain is regulated by phosphorylation events (reprinted from Lyons [38], with permission from Atlas of Genetics and Cytogenetics in Oncology and Haematology)

N-terminal regions, removal of both the calponin homology and the acidic domains results in constitutive activation of Vav GEF function [9, 17, 23, 26, 28, 29] (Fig. 6.1). Not surprisingly, removal of these domains activates the full oncogenic actions of Vav proteins [9, 17, 28, 29]. Poised as a critical intermediary in growth factor signaling, Vav3 couples the activation of growth factor-type receptors such as insulin-like growth factor receptor (IGFR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), insulin receptor and ROS receptor [16, 26, 30] to downstream signaling molecules including but not limited to Jun kinase, NF kappa B, MAPK, and Stat pathways [16, 26, 31]. Additionally, Vav3 is activated by Eph receptors [32]. Moreover, the activation of Vav3 is involved in integrin signaling [33–36].

Vav3 Isoforms

The mature Vav3 protein consists of 847 amino acids, with a molecular mass of approximately 98 kDa. The *VAV3* gene consists of 27 exons spanning 393.7 Kb on chromosome 1p13.3. Two isoforms (alpha and beta) are produced by alternative splicing, and a third transcript (Vav3.1) is thought to be derived from alternate promoter usage [37, 38]. The Vav3 alpha isoform is the canonical sequence and is derived from the full 27 exons. The other isoform, beta, differs from alpha in the amino terminus, where residues 1–107 MEPWKQCAQW…DLFDVRDFGK, are replaced by MQLPDCPCRAHLP. This beta isoform is produced from a unique exon 1 spliced to exons 4–27 [38, 39]. Additionally, the gene locus is complex and

could potentially produce up to 13 different isoforms resulting from alternative splicing and alternate promoter usage [38, 40] although to date, additional isoforms have not been identified. Vav3 is modified posttranslationally by phosphorylation. Phosphorylation site prediction identifies phosphorylation sites at T131, S134, Y141, Y173, S511, T606, and Y797 [38]. Sites residing in the N-terminal region regulate activation of Vav3 GEF function as discussed above [9].

The transcript variant, Vav3.1, consists of only the C-terminal SH3–SH2–SH3 domains (Fig. 6.1) and is derived from a unique exon 18 and exons 19–27 [38, 39]. This variant is speculated to act as a dominant-negative based on the Vav3.1 structure [37] (and our unpublished observations). Vav3.1 mRNA, like full-length Vav3, is expressed broadly in tissues and cell lines. However, there has been little information on whether a Vav3.1 functional protein is produced in vivo. Vav3.1 mRNA is downregulated in response to treatment of hepatocellular carcinoma Hep G2 cells with the chemotherapeutic agent astragaloside [41]. While the authors suggest that the antitumor actions of astragaloside are due to downregulation of Vav3.1, such a concept contrasts with the idea that Vav3.1 functions as a tumor suppressor through functional inhibition of full-length Vav3 [37] (and our unpublished observations). However, the published primer sequences used in this study are not predicted to differentiate between Vav3 and Vav3.1. Thus, it is unclear whether Vav3.1 is specifically down regulated.

Vav3 Physiologic Actions Revealed Through Genetic Knockout Studies

Immune system: Genetic knockout experiments have been instrumental in delineating the requirement for Vav3 in diverse physiological processes. Since Vav3 is highly expressed in cells of hematopoietic lineage, it is not surprising that Vav3 is intimately involved in processes pertaining to proper T and B cell function. Mice deficient in all three Vav proteins, as expected, show profound defects in both B and T cell signaling [42]. In fact, these mice completely lack functional T and B cells and are incapable of mounting either T cell-dependent or T cell-independent humoral immune responses [42]. Vav proteins have overlapping and redundant roles with respect to T and B cell function [42–44]. Additionally, Vav3 plays a role in wound healing responses through mediating beta 2 integrin responses in macrophages [36]. Vav3 null mice have significantly delayed healing responses of full thickness excisional wounds [36]. The defects are due in part to impaired regulation of integrin responses and release of transforming growth factor beta (TGFβ) from macrophages [36].

Nervous system: Given the role of small GTPases in mediating cytoskeletal changes and cell motility, it is not surprising that Vav3 is involved in neuronal axon growth and guidance. For example, NGF-induced neurite outgrowth in PC12 cells requires Vav3-mediated activation of Rac1 and involves upstream signaling by P13K [45]. Vav3 is also important for neuronal migration during development [46]. Mice

deficient in both Vav2 and Vav3 show defects in nerve regeneration, vascularization, and recovery following injury [47]. These findings suggest that delineation of Vav protein function in regenerating nervous tissue is key to designing interventions aimed at restoring nerve function following injury. Additionally, Vav3 is expressed at high levels in cerebellar Purkinje and granule cells [48]. Vav3 knockout mice exhibit defects in Purkinje cell dendrite branching, granule cell migration, and survival. Functionally Vav 3 deficient mice show abnormalities in motor coordination and gaiting suggesting the importance of Vav3 to proper cerebellar development and function [48].

Cardiovascular system: An important role for Vav3 in maintaining proper cardiovascular homeostasis is supported by experiments performed in Vav3 null mice. These mice exhibit many symptoms of cardiovascular dysfunction including tachycardia, hypertension, and cardiovascular remodeling. Consistent with these symptoms, Vav3 knockout mice also show a high degree of sympathetic tone manifested by elevated circulating levels of catecholamines and renin–angiotensin–aldosterone hyperactivity [49]. Over time, the changes result in progressive loss of both cardiovascular and renal homeostasis [49]. The mechanisms responsible for the alterations were not identified, but these studies clearly show the importance of Vav3 to the maintenance of normal cardiovascular function. A plausible mechanism for the cardiovascular defects was shown in Vav3 null mice where Vav3 is expressed in GABAergic neurons of the ventrolateral medulla, which regulates respiratory rate and sympathetic output. This medullary region contains sympathetic efferents, which are important in cardiovascular control and blood pressure regulation. Mice deficient in Vav3 exhibit reduced GABAergic transmission between the ventrolateral medulla and its postsynaptic targets resulting in deregulation of circulating levels of catecholamines and loss of normal blood pressure regulatory mechanisms [50]. The underlying mechanism responsible for the defects in GABAergic transmission is due to deficits in axon growth and guidance of the ventrolateral medullary GABAergic neurons [50]. This is in agreement with studies showing a role for Vav3 in proper axon growth and guidance during development. Interestingly, the aryl hydrocarbon receptor (Ahr) upregulates Vav3 expression in a variety of tissues [51]. In fact, the cardiovascular defects observed in Vav3 null mice are mimicked in Ahr-deficient mice, which also show defects in GABAergic transmission and synapse formation [51].

Mice deficient in both Vav3 and Vav2 show reduced endothelial migration in response to the presence of tumor cells [52]. Additionally Vav2 and Vav3 are essential for Eph A receptor-mediated angiogenesis both in vitro and in vivo [52]. A recent study showed reduced xenograft tumor growth of B16 melanoma or Lewis lung carcinoma cells in mice deficient in Vav2 and Vav3 that was secondary to impaired angiogenesis [53]. Vav3 is also involved in thrombus formation through effects on platelet activation. Consistent with a role for Vav3 in mediating integrin-dependent responses, Vav3 and Vav1 together are required for collagen exposure-mediated PLC activation in platelets. This signaling pathway occurs through the major platelet integrin alphaIIbetaIII [54]. Mice deficient in any one Vav protein show normal platelet activation responses. However, mice deficient in both Vav1 and Vav3 showed striking deficiencies in collagen-induced platelet activation and

aggregation, and this phenotype was not worsened in mice deficient in all three Vav proteins [54]. Furthermore, mice deficient in both Vav1 and Vav3 have reductions in thrombus formation in response to high levels of oxidized LDL [55]. This finding is significant with respect to interventions aimed at preventing vessel occlusion and emboli in patients with hyperlipidemias. Lastly, Vav3 is both necessary and sufficient for rat vascular smooth muscle cell proliferation. These effects occur through a Rac1-dependent mechanism, involving the effector Pak-1 [56]. Taken together these studies firmly establish the importance of the diverse roles of Vav3 in the cardiovascular system.

Skeletal system: Studies in osteoclasts support a role for Vav3 in mediating proper bone deposition. Specifically, Vav3-deficient osteoclasts exhibit abnormalities in actin cytoskeletal rearrangements, cell spreading, and resorptive activities [57]. Consistent with the actions of Vav3 on integrin signaling, the osteoclast defects are due to impaired integrin engagement [57]. Vav3-deficient mice have increased bone density and are refractory to PTH-mediated bone resorption [57]. Given the importance of osteoclast activities to the regulation of bone deposition and resorption processes, Vav3 may emerge as a viable drug target for osteoporosis.

Vav Proteins and Cancer

Members of the Vav family of proteins are deregulated in a variety of human cancers. In the simplest terms, many of the oncogenic actions of Vav3 are the result of activation of Rho family GTPases. Rho family members regulate cellular events such as invasion, angiogenesis, growth, and survival. Vav1 was initially identified as an oncogene through cell transformation and nude mouse tumorigenicity screens [10]. However, the truncated, oncogenic form has not been identified in human cancers to date [58]. Similarly, while truncated forms of Vav2 and Vav3 also transform cells, these forms have not been found in vivo.

The normal function of Vav1 is in the proper development and activation of T cells [44], and although there is redundancy between Vav1 and Vav2 with regard to T cell function and developmental processes, loss of Vav1 alone leads to a block at T cell developmental checkpoints [44]. Given this role, and its oncogenic potential, it was speculated that Vav1 would be involved in human leukemias and lymphomas. Somewhat unexpected, given that Vav1 is oncogenic, was the finding that Vav1 null mice have an increased incidence of aggressive lymphoblastic-like T cell leukemia/lymphomas [59]. It has been speculated that the deletion of Vav1 increases Notch1 signaling, which is well established as a promoter of human and murine T cell lymphoblastic lymphoma [44].

Consistent with an oncogenic role, Vav1 is implicated in neuroblastoma, pancreatic adenocarcinoma, and a variety of primary lung carcinomas [44]. In fact, Vav1 expression may be a prognostic biomarker of aggressive disease in pancreatic cancer [60]. Most studies that have looked at Vav2 participation in tumorigenic processes such as angiogenesis and cell survival have suggested redundant functions

with other Vav family members. As mentioned above, Vav2/3 deficiency resulted in a reduction in B16 melanoma ogenesis [53]. Lastly, Vav2 alone increases invasion of oral squamous cell carcinoma lines dependent on activation of the Rho GTPases, Rac1 and Cdc42 [61].

Vav3 Expression in Prostate Cancer

Vav3 protein was found to be overexpressed (relative to benign tissue) in approximately one-third of prostate cancer tumor specimens, but there was no correlation between Vav3 expression and Gleason score in this relatively small study [6]. A role for Vav3 in prostate cancer is supported by a study in which a constitutively active Vav3 mutant was targeted to prostate epithelium of transgenic mice. These mice developed the precursor lesion, high grade prostatic intraepithelial neoplasia, as well as adenocarcinoma [62].

Levels of Vav3 increase with prostate cancer progression in several different model systems. Elevation of Vav3 is relatively common in cell line models of castration-resistant prostate cancer. Vav3 expression increases with long-term androgen deprivation in the androgen-dependent, human prostate cancer cell lines, LNCaP and LAPC 4 [2, 3, 6]. Vav3 is expressed at elevated levels (compared to androgen-dependent cell lines) in several castration-resistant human cell lines including ALVA 31, PC3, DU145, and CWR-22Rv1 [2, 6]. Upregulation of Vav3 mRNA occurs in an LNCaP xenograft model system of castration-resistant disease. In these studies, LNCaP cells were introduced into immunocompromised mice, and small tumors were established. Mice were randomized into a control (intact) group or were castrated. In this model, tumors in the castrated mice do not grow for a number of weeks but eventually progress to castration resistance. Analysis of Vav3 mRNA revealed that levels were significantly elevated in recurrent tumors growing in castrated animals compared to the androgen-dependent tumors from intact mice [3]. Similarly, a study of the drug dutasteride (a 5alpha-reductase inhibitor) revealed that reducing DHT in mice bearing LuCaP 35 xenograft tumors resulted in upregulation of Vav3 compared to intact (androgen-replete) mice [7]. Marques et al. [8] recently examined gene expression changes occurring between the androgen responsive PC346C cell line and its therapy-resistant subline PC346DCC, which was derived by long-term culture in androgen-depleted media. In agreement with the findings of others, Vav3 was upregulated in the PC346DCC cell line as compared to the parental PC346C androgen responsive cell line. Interestingly, insulin receptor and epidermal growth factor receptor signaling networks were also deregulated in PC346DCC cells suggesting that Vav3 may exist in a phosphorylated (active) state in these castration-resistant cells [8]. Gene expression profiling of androgen-dependent vs. castration-resistant tumors of the *Nkx3.1;Pten* mutant mouse model of prostate cancer revealed upregulation of Vav3 mRNA following cancer progression [4, 5]. Together these data suggest that despite the heterogeneity

of prostate cancer, Vav3 overexpression is clinically relevant and may be a common mechanism of castration-resistant disease progression.

Importantly, we recently demonstrated that ectopic expression of Vav3 confers robust castration-resistant growth in a VCaP xenograft system [63]. In these experiments, Vav3 expression in the androgen-dependent human prostate cancer cell line, VCaP, results in continued rapid tumor xenograft growth after castration of the immunocompromised host animals whereas the control VCaP tumors exhibit a substantial delay in growth following castration.

The relevance of Vav3 overexpression to clinical progression is supported by studies that have examined levels of Vav3 mRNA in clinical specimens following neoadjuvant androgen deprivation therapy and in metastatic disease. Vav3 mRNA is upregulated in prostate cancer tumor specimens obtained from men undergoing androgen deprivation therapy compared to levels in primary tumors [64, 65] (and data deposited in public databases). Vav3 levels increase in late stage and metastatic prostate cancer [66]. The clinical relevance of Vav3 is illustrated by the finding that reduced levels of Vav3 are associated with fewer lymph node and bone metastases in men with prostate cancer [66]. In contrast, elevated Vav3 expression correlated with earlier biochemical failure (rising prostate-specific antigen) [66]. The authors suggest that Vav3 levels may be useful as a predictor of disease outcome [66]. In contrast, Marques et al. [8] examined Vav3 mRNA in clinical specimens obtained from radical prostatectomy or transurethral prostate resection by TaqMan real-time PCR. The specimens were derived from benign prostate as well as from patients with adenocarcinoma at different disease stages. Surprisingly, levels of Vav3 mRNA decreased with disease progression, with the lowest levels observed in metastatic tumors. The reasons for the discrepancy between the studies described above and those of Marques et al. [67] are not clear, but the authors of the latter study state that the probe used to identify Vav3 mRNA in the clinical specimens would not differentiate between Vav3 and Vav3.1. Since the Vav3.1 variant is thought to act as a dominant negative, these findings require follow-up studies using more selective probes for Vav3.

Vav3 as an Androgen Receptor Coactivator

Based on our initial finding that Vav3 levels increase during progression of prostate cancer cell models to castration resistance, a condition in which AR signaling is high, we examined the potential of Vav3 to modulate AR function [2]. Initial studies showed that Vav3 functions as a coactivator of the AR and very surprisingly that GEF function is not required for this action [2]. This finding was novel and was the first study to describe a GEF independent action of Vav3. Following the publication of this initial paper, data from other labs began to emerge supporting the concept that Vav3 is a novel AR coactivator [6]. Considerable information has been gained by mutational analysis of the functional domains of Vav3. Work from our laboratory

and others discerned that Vav3 enhances both ligand-dependent and ligand-independent AR activity [2, 3, 6, 63]. Although the mechanisms of each differ, the Vav3 pathways leading to enhanced AR activity in castration-resistant disease are not believed to be mutually exclusive [2, 3].

Vav3 Coactivation of Androgen Receptors: Ligand-Dependent Mechanisms

Vav3 enhances the activity of the AR in the presence of both saturating [2, 6] and subnanomolar levels of hormone [2]. This latter point is of clinical relevance as low levels of androgen are often present in castration-resistant disease [68]. We found that the Vav3 coactivator function occurs independent of GEF activity as three separate Vav3 mutant proteins, all lacking GEF activity, retain the ability to enhance AR activity in prostate cancer cell lines as measured by both reporter gene assays and target gene expression studies [2]. Other studies, however, concluded that the Vav3 DBL (DH) homology domain is required for AR coactivation [6]. However, these studies did not address the specific question of GEF function, since a Vav3 mutant lacking the entire DH domain did not coactivate AR. Although removal of the DH domain would certainly eliminate GEF function, the lack of AR coactivation by this Vav3 mutant could be due to loss of a structural component required for coactivation and not necessarily GEF activity.

Androgen inducible AR N–C interaction is essential for optimal AR chromatin binding and transcriptional activity [69]. Both Vav3 and a Vav3 GEF mutant greatly enhance androgen-mediated AR N–C interactions thereby facilitating AR transcriptional activity [63, 70]. This result further supports the GEF independence of Vav3 coactivation of AR. Sequential chromatin immuoprecipitation (CHIP) assays revealed that, in response to androgen, Vav3 and AR are present at the same transcriptional complexes at an AR target gene enhancer region [63]. This finding supports a novel nuclear role for Vav3. Vav3 mutational analysis illustrated that the Vav3 PH domain is required for ligand-dependent AR coactivation by Vav3 and that the PH domain is essential to facilitating nuclear localization [63]. More specifically, Vav3 mutants either lacking the PH domain or containing a mutation in an invariant tryptophan residue within the PH domain, which disrupts PH domain function, are greatly impaired in AR coactivation. The inability of the Vav3 PH mutant to coactivate AR was rescued by targeting the Vav3 PH mutant to the nucleus, suggesting that the critical role of the PH domain is to allow nuclear entry of Vav3. This experiment also demonstrates that Vav3 nuclear localization is essential for the enhancement of androgen-dependent AR activity by Vav3. Further confirmation of the importance of Vav3 nuclear localization in AR coactivation was shown by Vav3 subcellular localization analysis. Cell imaging studies indicated that wild type GFP-tagged Vav3 is localized to both the cytoplasm and nucleus; whereas, GFP-tagged Vav3 PH mutants (which are deficient in AR coactivation) are significantly excluded from the nucleus [63]. Vav3 nuclear and cytoplasmic localization

was also demonstrated by subcellular fractionation [70] (and our unpublished results). Vav3 nuclear localization is increased in response to DHT treatment, and the castration-resistant LNCaP AI cells, contain a larger fraction of nuclear Vav3 as compared to the parental LNCaP cell line [70]. The mechanisms by which Vav3 gains nuclear entry are largely unknown. The closely related Vav1 protein is a component of transcriptionally active complexes in immune cells [71]. Also, Vav1 is involved in RNA processing and regulates the amount of nuclear proteins involved in molecular complexes with DNA. It has been speculated that Vav1 affects nuclear transport of molecules required for modulation of mRNA production [72]. Interestingly, Vav1 was initially suspected to participate in nuclear processing or transcriptional modulation due to its domain organization and structure [10]. Vav1 contains two potential NLS motifs of which, one has been verified as mediating nuclear entry [71]. This functional NLS is well conserved in Vav3 and resides within the Vav3 PH domain. Surprisingly, mutation of this putative NLS in Vav3 had no effect on the AR coactivation function of Vav3 [63].

Studies examining a possible interaction between Vav3 and wild type AR have been conflicting, with one study finding minimal to no interaction [2] and another study demonstrating interaction dependent on the Vav3 DH and PH domains [70]. The discrepancy in these findings may reflect cell-specific differences as the studies, which failed to see a significant AR-Vav3 interaction were conducted in prostate cancer cells [2], whereas the studies which detected an interaction were conducted in Hela cells and used a truncated AR construct [70]. Vav3 does not appear to affect levels of full-length AR [2], and the C-terminal domains of Vav3 are not required for AR coactivation [2, 70]. LXXLL-type motifs, found on many nuclear receptor coactivators, serve as regions that interact with receptors, particularly in the ligand-binding domains, and facilitate receptor transcriptional activity [73]. Although Vav3 contains a conserved LXXLL motif and a related motif, neither was required for the AR coactivator function of Vav3 [63]. Vav3 actions on AR require the AR AF1 region, but not the AF2 regions, which is consistent with the lack of LXXLL motif involvement. Taken together these data suggest that Vav3 enhancement of ligand-dependent AR activity requires Vav3 nuclear localization, a process which is dependent on the Vav3 PH domain. Once in the nucleus, Vav3 is recruited to the enhancer regions of AR target genes where it facilitates AR transcriptional activity by unknown mechanisms. However, given the known adaptor functions of Vav3, it is possible that Vav3 bridges interactions important for full AR activation or aids in the nuclear transport of molecules required for full AR activation. Figure 6.3 depicts a model of Vav3 coactivation of AR in the presence of androgenic ligands.

Ligand-Independent Activation of Androgen Receptors by Vav3

Vav3 also enhances AR activity in the absence of hormone. This finding is important as it positions Vav3 as a key player in castration-resistant prostate cancers. Results from our laboratory showed that, in contrast to the ligand-dependent actions

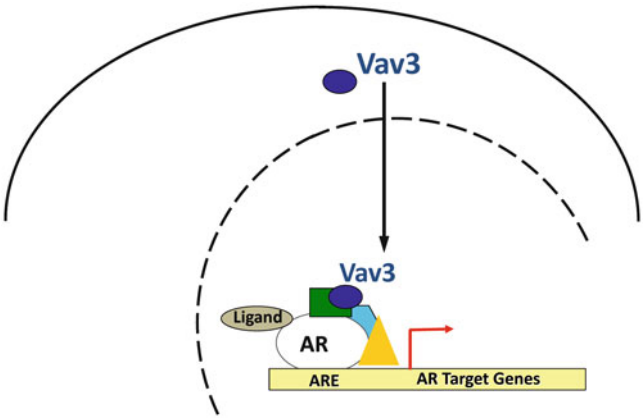

Fig. 6.3 Ligand-dependent AR coactivation by Vav3: Nuclear Vav3 is co-recruited with liganded AR to AR target genes and potentiates AR transcriptional activity

of Vav3 on AR, ligand-independent activation of AR by Vav3 requires GEF function and the Rho GTPase Rac1. Interestingly, Vav3 is coupled to many of the same growth factor receptor pathways that are involved in ligand-independent AR activation such as those mediated by IGF, EGF, and PDGF receptors [30]. In addition to the enhancement of AR transcriptional activity by a constitutively active variant of Vav3, wild type Vav3 could also enhance ligand independent activation of AR by the growth factors IGF and EGF [3, 70]. Since Vav3 is activated by both the EGF and IGF receptors, Vav3 may facilitate growth factor signaling mechanisms in castration-resistant prostate cancer. Data from these studies also revealed the strict requirement for the RhoGTPase Rac1 and the involvement of the MAPK/ERK pathways, as specific inhibitors of this pathway abolished the ligand independent actions of Vav3 on AR [3]. Additional studies have also suggested the involvement of the PI3Kinase pathway [6, 70]. Taken together, these studies indicate that Vav3 coactivates AR in the presence of androgen (subnanomolar to saturating doses) (Fig. 6.3) and also that growth factor-activated Vav3 enhances AR activity in the absence of androgen (ligand-independent activation) (Fig. 6.4).

Another recently identified mechanism of ligand-independent AR signaling in prostate cancer is through the expression of constitutively active AR splice variants that lack the hormone-binding domain (reviewed in [74–76]). These variants are expressed in castration-resistant human prostate cancer and represent a key mechanism by which advanced tumors may evade therapy directed at the hormone-binding domain [77–80]. We found that Vav3 enhances AR splice variant signaling, which is to our knowledge, the first detailed example of AR splice variant coactivation [81]. In addition, we found that Vav3 and the prevalent AR splice variant, AR3 (aka AR-V7) are critical for castration-resistant prostate cancer survival, proliferation, and ligand-independent AR activity. In contrast to full-length AR, Vav3 binds to AR3 and increases nuclear levels of AR3 providing a mechanism for the actions of this novel AR splice variant interacting partner.

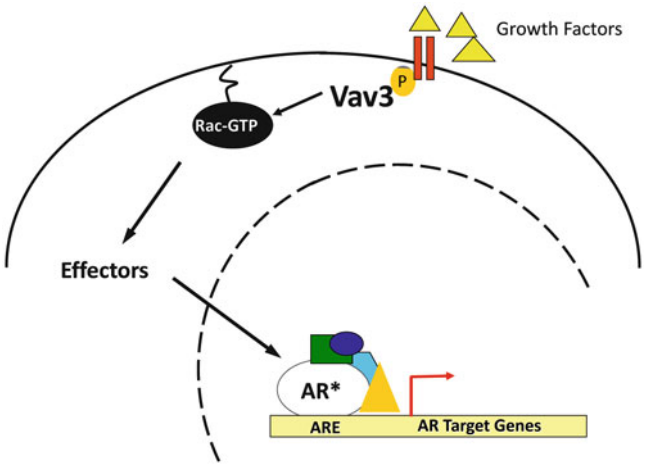

Fig. 6.4 Ligand-independent AR activation by Vav3: As a Rho GTPase GEF, Vav3 is phosphorylated and activated by growth factor receptors leading to formation of Rac1-GTP and AR activation in the absence of hormone

Involvement of Vav3 in Other Cancers

Breast cancer: Data have emerged showing a role for Vav3 in breast cancer. Vav3 expression is elevated in up to 81% of human breast cancer specimens [82]. Vav3 enhances ER alpha activity, and this activity requires both the DH domain [82] and Vav3 activation of the small GTPase Rac1 [83]. Rac1 increases ER alpha levels, and inhibition of Rac1 selectively inhibited proliferation of ER-positive breast cancer cell lines [83].

Gastric cancer: A recent study examined 167 cases of gastric cancer over a number of years and found that in clinical specimens, overexpression of Vav3 was correlated with invasion, formation of distant metastasis, and poor disease-free survival [84]. The authors concluded that Vav3 expression is an independent prognostic marker for gastric cancer [84] and suggested that Vav3 is a rational drug target for the treatment of gastric cancer. Earlier studies of gastric cancer hinted at the involvement of Vav3. For example, downregulation of RUNX3, a member of the runt domain-containing family of transcription factors that has tumor suppressive actions, has been implicated in promoting human gastric carcinogenesis. Studies of gastric cancer showed that Runx3 expression resulted in the downregulation of a number of genes including Vav3 thereby providing a potential link between Vav3 expression and gastric malignancy [85].

Glioblastoma: Vav3 is upregulated in glioblastoma as compared to non-neoplastic or lower grade gliomas [86]. Downregulation of Vav3 by siRNA reduced glioblastoma invasion and migration. Moreover, upregulation of Vav3 was shown to be an indicator of poor patient survival [86], thus providing another indication of the importance of Vav3 levels to clinical outcome.

Lymphoma and leukemia: Given the robust expression of Vav3 in cells of hematopoietic lineage, its role in lymphomas and leukemias is not surprising. Vav3 is overexpressed in hepatosplenic T cell lymphoma [87] and has been implicated in nucleophosmin anaplastic lymphoma kinase (NPM–ALK) fusion protein signaling. This oncogenic protein has constitutive tyrosine kinase activity. Vav3 is activated via its SH2 domain by Npm–Alk resulting in subsequent Rac1 activity. Vav3 and Rac1 are required for NPM–ALK-mediated motility and invasion [88]. Additionally a role for Vav3 has been established in leukemias. Vav3 was shown to be required for efficient leukemogenesis by p190-BCR-ABL. Also, Vav3 overexpression could compensate for deficiency of both Vav1 and Vav2 in p190-BCR-ABL-mediated transformation in B cell progenitor cells [89].

Conclusions

Vav proteins participate in the promotion of a wide variety of cancers ranging from hematologic malignancies to solid tumors. Although predicted, based on the cloning of an oncogenic fragment of Vav1, no activating mutations of Vav family members have been identified in human cancers to date. Instead, the oncogenic mechanisms of Vav proteins appear to involve overexpression of these proteins leading to the stimulation of proliferation, migration, angiogenesis, and invasion. While these oncogenic processes require Vav activation of Rho GTPases, we demonstrated that Vav3 also exerts oncogenic effects on prostate cancer in a GEF-activity-independent manner through potentiation of androgen-inducible AR activity or through enhanced AR splice variant constitutive signaling. While all Vav proteins can serve as coactivators of AR in transfection experiments [63], Vav3 appears to be uniquely involved in prostate cancer as levels of this Vav family member are specifically increased in prostate cancer, particularly in castration-resistant disease ([2, 3, 6, 63, 64, 66] and publically available databases). Castration-resistant prostate cancer cells appear to be particularly adept in exploiting Vav3 signaling to promote proliferation, survival, and metastasis. Vav3 is a versatile enhancer of AR by both ligand-dependent and ligand-independent mechanisms and as such impacts all currently described pathways of AR reactivation in advanced, castration-resistant disease [90]. In particular, expression of Vav3 may permit resistance to commonly used prostate cancer therapies that are directed at the ligand-binding domain of the AR. Even newer modalities including abiraterone, which blocks CYP17 in the synthesis of testosterone, may conceivably be overcome by ligand-independent activation of AR by Vav3. Ligand-independent activation of AR is promoted by Vav3 through effects on the Rho GTPase Rac1 [3] and through recently described stimulation of AR splice variants [81] that lack ligand-binding domains and are constitutively active. These multifaceted actions of Vav3 on AR indicate that new therapies will need to target all mechanisms of Vav3 including activation of Rac as well as direct Vav3 effects in the nucleus. While promising Rac inhibitors are in development [91, 92], inhibiting Vav3 nuclear actions is an equally laudable challenge for the future.

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Chapter 7
Transforming Growth Factor-Beta in Prostate Cancer

David Danielpour

Abstract TGF-βs are 25 kDa dimeric proteins that function through autocrine, paracrine, and endocrine mechanisms to regulate a diverse array of cellular and physiological processes in numerous tissues. Deregulation of TGF-β signaling is involved in the pathophysiology of prostate cancer. Central to normal prostate epithelial and stromal cell growth control mechanisms, TGF-β functions as a tumor suppressor and an important regulator of androgenic responses. Particularly striking, androgen withdrawal activates multiple components of the TGF-β signaling pathway in the normal prostate, which then partake in the ensuing apoptotic cell death response. Multiple discreet alterations in TGF-β signaling responses occur during the process of carcinogenesis and tumor progression, which contribute to the development of both metastatic disease and ultimately to castrate-resistant prostate cancer (CRPC). Despite its seemingly straightforward role as a tumor suppressor in the normal prostate, there is accumulating evidence supporting that the function of TGF-β "switches" to a tumor promoter during carcinogenesis/tumor progression. This represents what has now been coined the "TGF-β paradox," the molecular and physiological basis for which remains incompletely defined. The TGF-β paradox also imposes inevitable complexities in therapeutic strategies involving TGF-β. However, recent advances provide significant promise for TGF-β as a prognostic marker and therapeutic target of prostate cancer (PCa).

This chapter provides a current overview of key components of the TGF-β signaling pathway. Starting with some historical perspective, the chapter highlights fundamentals of the TGF-β ligand structure, regulation of expression, storage, and

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activation. Next illustrated are the nuts and bolts of TGF-β receptor and Smad structure and function. A thorough perspective and mechanistic insight is provided on the current understanding in the field of TGF-β in normal and malignant prostate and state-of-the-art progress on TGF-β-based preclinical and clinical therapeutic opportunities.

Introduction

Transforming growth factor-beta (TGF-β) was discovered about three decades ago, first identified in a distinct peptide fraction of sarcoma growth factor (SGF) activity secreted from Moloney sarcoma virus-transformed NIH-3T3 fibroblasts [1]. The first chromatographic fraction of SGF was a 6 kDa peptide called transforming growth factor-α (TGF-α) and the second fraction (25 kDa) was TGF-β, named for their ability to promote phenotypic malignant transformation, as assayed by anchorage-independent growth in soft agar [2–4]. TGF-β was later isolated from many normal tissues [5–9] and found to be a multifunctional regulator endowed with potent growth inhibitory activity on normal epithelial cells [10–12]. Since then the TGF-β field has grown exponentially, with >58,000 citations in PubMed. Only ~1,000 of those are on prostate, putting into perspective the broad spectrum of TGF-β's multifunctional and multitissue activity. Indeed, TGF-β, its receptors and canonical signaling mediators are found to various degrees in essentially all normal tissues and involved in numerous physiological and pathological processes [13–15].

TGF-β Signal Transduction

TGF-β Ligands

Mammals are endowed with three isoforms of TGF-β, namely TGF-β1, TGF-β2, and TGF-β3, each a 25 kDa secretory homodimer encoded by a unique gene [16]. Two other isoforms, TGF-β4 and TGF-β5, have been reported in birds and reptiles, respectively [17, 18]. TGF-βs share 64–82 % sequence homology and nine conserved cysteines and are each synthesized with a long N-terminal pro-peptide and a C-terminal mature growth factor domain [16]. Within cells, the precursor sequence forms a disulfide-bonded homodimer that is proteolytically cleaved by convertases (furins) to form a mature dimeric TGF-β and a 75–80 kDa latency-associated peptide (LAP) [19–21]. Upon secretion, each TGF-β remains associated to its LAP as a small latent complex (SLC) [22]. One of a number of unique properties of each mature TGF-β is exquisite amino acid sequence conservation. For example, there is 100 % identity between TGF-β1 in human, monkey, cow, and pig. TGF-βs share 30–40 % sequence homology to other functionally distinct groups of proteins that comprise the TGF-β superfamily; these include the bone morphogenetic proteins

(BMPs), Mullerian inhibiting substance (MIS), inhibin-α, activins, growth differentiation factor (GDF), and nodal, among others [16].

TGF-βs are secreted as 100–105 kDa SLCs, which often associate to 125–240 kDa latent TGF-β-binding proteins (LTBPs) that are believed to function as a latent reservoir of TGF-β in extracellular compartments [19]. There are four LTBPs, each encoded by a distinct and differentially expressed gene. TGF-βs are activated through various enzymatic (plasmin, metalloproteinases, kallikrein) and nonenzymatic (i.e., thombospondin-1, integrins) mechanisms that still remain to be fully resolved [23–27]. Because free LAP can bind to and inactivate TGF-β, activation of TGF-β must require the ultimate sequestration or structural modification of LAP. A recent crystallographic study of TGF-β large latency complex (LLC) reveals that binding of $\alpha_v\beta_6$ integrin to the LAP segment of this complex induces a conformation change that causes the release of active TGF-βs 1 and 3 [28]. Prostate-specific antigen (PSA), which is elevated in PCa, has been shown to activate TGF-β2 but not TGF-β1 by selectively cleaving TGF-β2 LAP [29]. While each TGF-β isoform is functionally indistinguishable in most in vitro systems, they each have unique tissue expression patterns and distinct functions in vivo [30, 31]. TGF-β1 homozygote null knockout mice have a relatively short lifespan due to autoimmunity [32], whereas TGF-β2 and TGF-β3 homozygote null mice each develop a cleft palate and TGF-β2 and TGF-β3 homozygote double knockout mice exhibit early embryonic lethality [32–34].

Each TGF-β is differentially expressed and activated during development and upon various cellular stresses. Unique sets of promoter response elements and post-translational control mechanisms provide the basis for such control [35]. In general, TGF-β1 expression is elevated by signals that promote cell growth and proliferation, whereas TGF-βs 2 and 3 are induced by differentiation and growth arrest signals. For example, epidermal growth factor (EGF), platelet growth factor (PDGF), insulin, insulin-like growth factor (IGF-I), H-Ras, C-Src, or serum typically induce the expression of TGF-β1 and suppress that of TGF-β2 [36–38], whereas serum-withdrawal, retinoic acid, vitamin D, and anti-estrogens favor the expression of TGF-βs 2 and 3 [39–42]. An exception is that TGF-β1 robustly induces its own expression (autoinduction), mediated partly through AP-1 [43]. Growth suppressive signals generally promote the activation of the retinoblastoma protein (Rb), which induces TGF-β2 expression through facilitating the interaction of ATF2 to the TGF-2 promoter [44].

TGF-β Receptors

TGF-β receptors were first identified by cross-linking ^{125}I-TGF-β1 to cell surface proteins on intact cells [45]. Three unique TGF-β-binding proteins (shown to be transmembrane proteoglycans) were identified on most cell types (named TβRI [55 kDa], TβRII [75 kDa], and TβRIII [280 kDa]) (Fig. 7.1) [46–48]. Other less characterized cell surface-binding proteins that bind all three TGF-βs (TβRIV

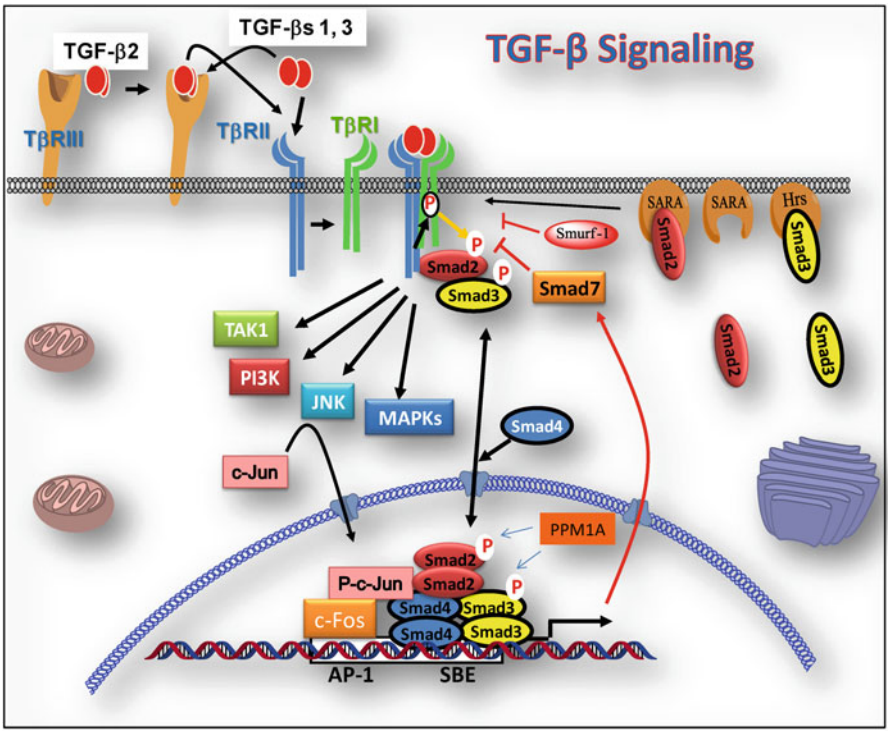

Fig. 7.1 This is a simplified schematic representation of TGF-β signaling, starting from ligand–receptor interactions through its control of gene expression. Shown are a number of key components involved in its canonical (Smads) and non-canonical (TAK1, PI3K, JNK, MAPKs) pathways. Also shown is how TGF-β signaling is inhibited by Smad7 (induced by TGF-β), by ubiquitin-mediated degradation of TGF-β receptors and Smads (by Smurfs) and by dephosphorylation of R-Smads by the metallophosphatase PPM1A

[60–64 kDa proteoglycan], TβRV [400 kDa proteoglycan]) or selectively bind to a given TGF-β isoform have also been identified in specific tissues/cells [49–52]. While TβRI is critical for mediating TGF-β1 responses, it is unable to bind directly to TGF-β1. TGF-β1 associates with TβRII, causing a conformation change in TβRII that promotes the recruitment of TβRI to form a ligand-receptor heteromeric complex consisting of a single dimeric TGF-β, two TβRIIs and two TβRIs (Fig. 7.1) [53–55]. Following isolation and cloning of the receptor genes [56–58], TβRI and TβRII were shown to be transmembrane glycoproteins with a cysteine-rich extracellular ligand binding domains and a serine–threonine kinase domain [48, 49]. Unlike TGF-βs 1 and 3, TGF-β2 is unable to bind directly to either TβRII or TβRI. The biological responses of TGF-β2 in cells expressing endogenous levels of TβRII and TβRI require TβRIII (also called betaglycan), a heavily glycosylated transmembrane protein with a long cysteine-rich extracellular domain and a short intracellular segment that lacks a kinase domain [59]. In contrast to TβRII, TβRIII binds to all

three mammalian isoforms of TGF-β with relatively high affinity (Kd ~0.1 nM) and can enable or enhance the biological activity of all three mammalian isoforms of TGF-β by delivering them to TβRII [59]. The extracellular domain of TβRIII is shed by cells under certain conditions, such as inflammation and during carcinogenesis, and its soluble form antagonizes TGF-β responses [60].

Once in a TβRII–TβRI–ligand complex, the TβRII kinase phosphorylates TβRI at a juxtamembrane site, known as the GS box, promoting the activation of TβRI kinase (Fig. 7.1). The immunophilin, FKBP12, binds to the Leu–Pro sequence near the phosphorylation sites of TβRI [61, 62] and prevents ligand-independent activation of TβRI by TβRII but not their physical association [63, 64].

Smads: Structure, Regulation, and Transcriptional Control

Smads comprise a family of structurally highly conserved proteins, with an N-terminal MH1 DNA-binding domain and C-terminal MH2-protein interaction domain, separated by a poorly conserved linker [65]. To date, eight different Smads have been identified in mammals. They fall into three distinct functional groups: (1) receptor-activated Smads (R-Smads: Smads 1, 2, 3, 5, 8), (2) mediator Smad (Smad4), and (3) inhibitor Smads (Smads 6 and 7) [66–69]. The MH1 domain of Smad2 differs from those of Smads 1, 3, 4, 5, and 8 mainly by a 30 amino acid insertion sequence that interferes with direct binding to consensus Smad-binding element (SBE) [70, 71]. Smads are further subclassified into two distinct groups with respect to the ligand subfamily they serve. Smads 2, 3, 4, and 7 belong to the TGF-β subfamily, and Smads 1, 5, 8, and 6 belong to the BMP subfamily, whereas Smad4 serves both subfamilies. While there is a significant amount of receptor specificity for the activation of R-Smads, this specificity is not absolute. The L45 loop of TβRI and L3 loop of Smad confer specificity of interactions of R-Smads to various forms of TβRIs (Alks 5, 4, and 7) [72–74]. Smads 2 and 3 are recruited to TβRI with the aid of accessory proteins such as the membrane anchor FYVE domain containing proteins, *S*mad *a*nchor for *r*eceptor *a*ctivation (SARA) [75] and Hrs/Hgr [76] (Fig. 7.1). The TβRI kinase then phosphorylates the carboxyl SSXS domains of those Smads, a modification which promotes R-Smad homodimerization, allowing them and Smad4 to be imported to the nucleus through association with Importin-β upon exposure of conserved nuclear localization signal (NLS) motifs [55, 67, 77–79]. Prior to activation by TβRI, Smads 2 and 3 are auto-inhibited through an association between their N-terminal and C-terminal domains and associated to microtubules [70, 80]. In contrast to Smads 3 and 4, the nuclear targeting sequence of Smad2 is located in the C terminus and masked by SARA [70, 79, 81].

Various kinases, including Erk, JNK, p38 MAPK, and Cdk4 modulate TGF-β signaling through selective phosphorylation of R-Smad linker [82]. Once in the nucleus, Smads 3 and 4 bind directly to consensus SBE (i.e., GTCTAGAC) located in target gene promoters or/and enhancers or bind indirectly to other promoter elements via association with various transcription factors, ultimately modulating the

transcription of a variety of TGF-β target genes that mediate their various activities [83–85]. However, the binding of Smads to SBE in target promoters and enhancers is relatively weak and thus depends predominantly on their interaction with other transcription factors that bind to response elements in proximity to SBEs [86]. Smads 2, 3, and 4 thus partner with numerous proteins, which include transcription factors and coregulators such as P/CAF, MSG1, SNIP, p300/CBP, TGIF, HDAC, Sno, Ski, AP-1, and steroid hormone receptors [83, 87] to regulate gene expression. Moreover Smads 2 and 3 are also activated by receptors belonging to other members of the TGF-β superfamily (i.e., activin and inhibin). TGF-β receptors also activate non-Smad signaling cascades through mechanisms that remain inadequately understood [88, 89].

TGF-β signaling is highly dynamic, as it is quickly turned off through the collaborative activity of Smad7, ubiquitin-mediated degradation of TGF-β receptors and Smads through HECT, Smurfs, ROC-1, and Arkadia [90] and the inactivation of R-Smads by the phosphatase PPM1A [91] (Fig. 7.1).

General Functions of TGF-βs

TGF-βs play key roles in the regulation of numerous normal cellular, physiological, and developmental processes, which include control of cell proliferation, differentiation, apoptosis, autophagy, senescence, cell migration, invasion, chemotaxis, cell adhesion, extracellular matrix deposition, embryogenesis, mesoderm induction, bone and muscle development, angiogenesis, immune regulation, inflammation, wound healing, and epithelial–mesenchymal transition (EMT) [13, 92]. TGF-βs function in a broad spectrum of cell types, tissues, and organ systems, and its various activities are context specific [93]. With respect to proliferative effects, normal epithelial cells typically respond to TGF-β by growth arrest and apoptosis, whereas TGF-β promotes the survival and growth of stromal fibroblasts and neuronal cells. Mechanisms of growth arrest by TGF-β are Smad dependent and occur through downregulation of various cyclins, cyclin-dependent kinases, and upregulation of cyclin-dependent kinase inhibitors [94, 95]. TGF-β can also suppress growth via downregulation of cdc25A, through recruitment of HDAC by E2F-p130 [96], and by downregulation of the proto-oncogene c-Myc, which also frees its binding partners Max and Miz-1, the latter of which then transcriptionally activates $p15^{INK4b}$ [97–99].

TGF-β induces apoptosis in a variety of cells, likely through multiple related mechanisms. The activation of both Smads and AP-1 is crucial for such apoptosis in certain scenarios [85]. TGF-β-induced apoptosis occurs by the activation of various caspases through both the intrinsic and extrinsic pathways [100–104]. Mechanisms of apoptosis by TGF-β involve the induced expression of pro-apoptotic BCL2 family members, down-regulation of anti-apoptotic members of this family [105, 106], and the release of cytochrome c from mitochondria followed by the activation of caspases −9 and −3 [105]. Other effectors include DAP kinase [107], a MAP kinase

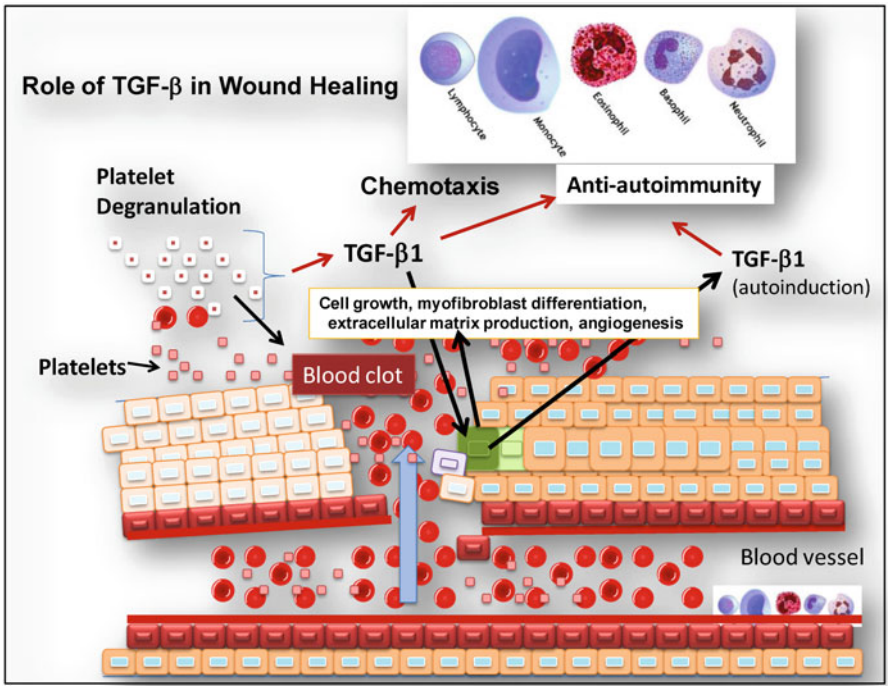

Fig. 7.2 Schematic representation of the role of TGF-β in wound healing is illustrated here to better under understand how the deregulated overexpression of TGF-β in cancer could promote tumor growth and progression through its various functions during injury

member (TAK-1) [108], Daxx [109], NF-kB [110], and Smad7 [111]. Overall, the specific mechanisms of TGF-β growth arrest and apoptosis generally vary with cell type and tissue.

The multiple and seemingly conflicting functions of TGF-β in cancer can perhaps best be understood by comparison with the process of tissue injury and wound repair (Fig. 7.2). Platelets represent one of the most abundant sources of TGF-β1, which is quickly released and activated at the wound site upon platelet degranulation [112]. TGF-β is chemotactic for monocytes, macrophages, and fibroblasts and stimulates dermal fibroblasts to proliferate and differentiate into myofibroblasts, which are programmed to express various extracellular matrix proteins such as fibronectin and type I collagen [113]. Autoinduction of TGF-βs then amplifies this signal. Although TGF-β functions as a chemoattractant for immune cells to combat microbial infection, it simultaneously functions as a potent immunosuppressant to curtail autoimmunity due to tissue damage [114]. TGF-β1 knockout mice exhibit delay and deficiency in wound healing [115]. Paradoxically, Smad3 knockout mice show accelerated wound healing by increased rates of re-epithelialization and significantly reduced local inflammation, suggesting that the reparative processes occur largely though a Smad3-independent mechanism [116].

Functions of TGF-β in the Normal Prostate

Both stromal and epithelial compartments of the normal prostate express all three TGF-β isoforms [117], TβRI, TβRII, TβRIII [118, 119], along with Smads 2, 3, and 4 [120]. Immunohistochemical analysis showed equal expression of TGF-β1 in stromal and epithelial cells, whereas staining for TGF-βs 2 and 3 is greater in the epithelial relative to the stromal compartments [117]. Basal levels of TGF-βs expressed in the normal prostate are seemingly low relative to certain other normal tissues [121], and exogenous TGF-β1 placed on the ventral prostate of an intact rat promotes regression of glandular prostate cells, yielding a response similar to that of castration [122]. Likewise, exogenous addition of TGF-β1 to ventral prostate organ cultures induces death of glandular epithelial cells [122]. TGF-β is also important in prostate stromal cell function and in controlling stromal–epithelial cell interactions. Normal adult prostatic fibroblasts transdifferentiate to smooth muscle cells following TGF-β1 treatment [123]. In vivo evidence supporting TGF-β's critical role in normal prostate stromal–epithelial interactions is clearly illustrated by a study in which TGF-β signaling was selectively knocked out in fibroblasts by cre–lox targeted with a fibroblast-specific promoter; the result was development of prostate intraepithelial neoplasia (PIN) [124]. TGF-β indirectly establishes negative growth control of prostate epithelium through adjacent fibroblasts likely by suppressing the production of hepatocyte growth factor [124]. Production of active TGF-β by prostate basal epithelial cells promotes their differentiation into luminal epithelium cells [125], and through a paracrine mechanism TGF-β made by basal epithelial cells selectively suppresses growth and induces apoptosis of luminal variants that are highly sensitive to apoptosis by TGF-β [126]. Thus, disruption of the ability of TGF-β to control such cell–cell interactions may promote uncontrolled proliferation and lead to malignant transformation.

Connections Between Androgen and TGF-β Signaling

The luminal epithelium of the prostate, which expresses high levels of androgen receptors (AR), is exquisitely dependent on a continuous source of gonadal androgens for growth and viability. Surgical castration or administration of AR antagonists rapidly leads to regression of this epithelium through apoptosis [127]. Owing to partial retention of androgen dependency in early-stage metastatic PCa, a substantial effort has been invested in elucidating the mechanisms for such dependence [128]. Studies first conducted in 1989 showed that surgical castration of rats robustly (>10-fold) elevated the expression of TGF-β1 mRNA only after 1 day, which preceded the onset of apoptosis [118]. Subsequent studies revealed that androgen withdrawal also promotes the elevation of TGF-βs 2 and 3, TβRI, and TβRII [129], promotes activation of Smads 2 and 3 in rodents [120], and elevates TGF-β1 and TβRII in clinical PCa specimens [130]. These results together with other in vivo [122] and in vitro [131, 132] studies suggest a central function of TGF-β in regression of the prostate by androgen ablation.

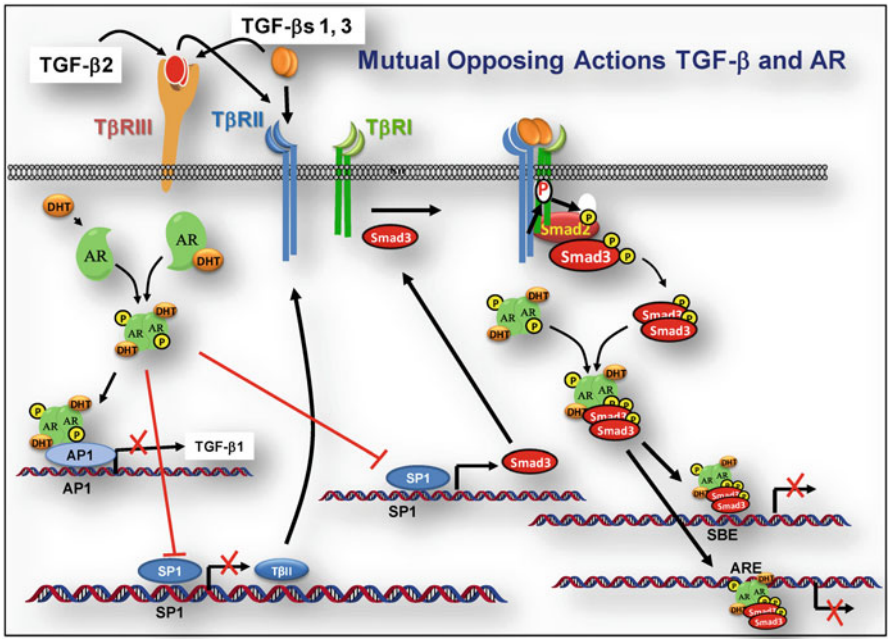

Fig. 7.3 Schematic illustration of the mutual opposing actions of TGF-β and androgen signaling at the transcriptional level in the prostate epithelial cells. Following the activation by DHT, AR is shown to repress TGF-β signaling through inhibiting the autoinduction of TGF-β1, repressing the transcription of TβRII and Smad3. AR also binds to Smad3, which further represses transcriptional response of Smad3 (through SBE) and transcriptional responses of androgen through androgen response elements (ARE)

Various studies have interrogated the mechanistic basis for the androgenic control of TGF-β responses, as well as the role of TGF-β in androgenic responses (see Fig. 7.3). Androgens promote growth of the nontumorigenic NRP-152 rat prostatic epithelial cells at least partly by suppressing the expression of autocrine TGF-βs 1, 2, and 3 [133]. Androgens can also suppress the auto-induction of TGF-β1 in the LNCaP androgen-dependent human prostatic adenocarcinoma cell line [134].

Smad3 is a common molecular interface between androgen and TGF-β signaling. AR can bind to Smad3 (but not to Smads 2 or 4), mediating either inhibition [135, 136] or enhancement [137] of androgenic responses, particularly AR-dependent transcriptional control. The binding of AR to Smad3 may also provide the molecular basis by which TGF-β stifles the translocation of AR from the cytoplasm to the nucleus in prostatic stromal cells, blocking androgenic responses [138]. In addition, binding of AR to Smad3 represses transcriptional responses of TGF-β1 [134]. Electrophoretic mobility shift assays using GST-AR and GST-Smad3 fusion proteins support the concept that the ligand-binding domain of AR and the MH2 domain of Smad3 are critical to tethering AR to Smad3, and in the presence of DHT results in a Smad3 complex with reduced affinity for SBE [134].

In addition to forming a molecular complex with Smad3, AR-DHT represses the expression of Smad3 protein and mRNA, as demonstrated in cells expressing endogenous AR (in LNCaP and VCaP), and by enforced expression of AR (in AR-deficient PCa cells lines, DU145 and NRP-154) [139]. In contrast, levels of Smad2 and 4 were essentially unaltered by DHT, although a small loss in the expression of Smad2 could be seen by 2 days of treatment, suggesting that androgens differentially regulate expression of Smads 2 and 3 in PCa cells. Experiments using various Smad3 gene promoter–luciferase reporter constructs transiently transfected into the above cell lines show that androgens repress Smad3 promoter activity [139].

A third mechanism by which AR affects TGF-β responses is through downregulating the expression of TβRII, which has been demonstrated in a number of AR-positive PCa cell lines or by overexpression of AR in AR-negative PCa cell lines [140]. In contrast, TβRI is not significantly altered by AR-DHT. Similar to Smad3, DHT represses the TβRII promoter [140] and TGF-β signaling responses, including the repression of cell growth, induction of apoptosis, downregulation of cyclin Ds, survivin, Bcl-xl, induced expression of PAI-1, and activation of Rb in PCa cells expressing either endogenous AR or exogenous AR [139, 140].

The LNCaP cell line is weakly responsive to TGF-β, as it expresses very low levels of TβRII [141]. Using LNCaP cells made highly responsive to TGF-β by enforced overexpression of TβRII, Kyprianou's laboratory has demonstrated that androgens can paradoxically enhance the cell death pathway induced by TGF-β [142, 143]. The molecular basis for this counterintuitive effect remains to be defined. Our results suggest this discrepancy may be clarified by the enforced overexpression of TβRII in those cells, which does not experience the repressive effects of AR through the endogenous TβRII promoter. Indeed, we show that DHT elevates the levels of exogenously expressed TβRII in LNCaP cells (unpublished), and this may be related to the mechanism by which EGF stabilizes TβRII mRNA in those cells [144]. Furthermore, we provided data illustrating that androgens block the ability of TGF-β to induce cell death only when TβRII is moderately overexpressed, more likely within physiological levels. However, robust overexpression of TβRII reversed the ability of DHT to protect NRP-154-AR cells from TGF-β-induced cell death [139]. In fact, our preliminary results suggest that DHT may stabilize the levels of ectopically overexpressed TβRII (unpublished), while simultaneously downregulating the expression of Smad3 (through promoter suppression) and inhibiting TβRII promoter activity. These latter results are consistent with AR-mediated protection of activin-induced death in LNCaP cells expressing endogenous activin receptors [145].

Control of Apoptosis Induced by TGF-β in Prostate Epithelial Cells

Defects in the mechanism underlying TGF-β-induced apoptosis during carcinogenesis are likely to ablate the tumor repressor function of TGF-β1. The NRP-152 and NRP-154 rat prostate epithelial cell lines, which were derived from the

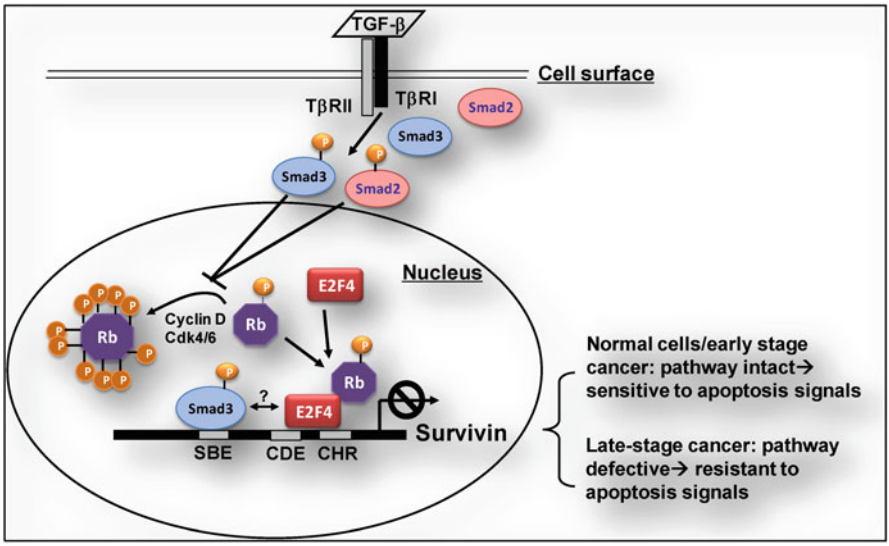

Fig. 7.4 Schematic model of Survivin regulation by TGF-β and its implication in prostate cancer. TGF-β ligand-bound receptors activate R-Smads, which activate Rb and Rb-like proteins by inducing their hypophosphorylation. Activated Rb (and Rb-like proteins) then binds to E2F4 to form a repressor complex that adheres to the CDE/CHR elements in the proximal region of the Survivin promoter. In normal prostate epithelial cells, the TGF-β/Survivin regulatory axis is intact and maintains Survivin levels. However, this axis is deregulated in prostate cancer, promoting increased levels of Survivin and resistance to anti-androgens and chemotherapy (adapted from Yang J, Song K, Krebs TL, Jackson MW, Danielpour D. Rb/E2F4 and Smad2/3 link survivin to TGF-beta-induced apoptosis and tumor progression. Oncogene 2008;27(40):5326–5338, with permission from Nature Publishing Group)

pre-neoplastic prostate of the Lobund–Wistar rat model of prostate carcinogenesis and are exquisitely sensitive to TGF-β-induced apoptosis [146], are suitable models for mechanistic studies of TGF-β-induced apoptosis [131]. The induction of apoptosis by TGF-β in NRP-154 cells requires the downregulation of the anti-apoptotic protein Bcl-xL, leading to mitochondrial release of cytochrome c and subsequent activation of caspases −3 and −9 [105]. Smads 3 and 2 have been shown to be critical to the induction of apoptosis in rat prostate epithelial cells, although the relative importance of each appears cell-type specific [147, 148]. TGF-β also rapidly downregulates a select member of the inhibitor of apoptosis protein (IAP), Survivin, in NRP-152, NRP-154, and the nontumorigenic human prostate cell line RWPE-1, but not in PC3 and DU145 prostate carcinoma lines [147]. TGF-β downregulates the Survivin promoter through a Smad2/3-dependent mechanism involving the hypophosphorylation of Rb and the subsequent association of the Rb/E2F4 complex to CDE/CHR elements in the proximal region of the survivin promoter (Fig. 7.4). Survivin silencing and overexpression experiments support a key role of this regulatory mechanism in the induction of apoptosis by TGF-β [147]. Disruption of this regulatory mechanism in advanced PCa may contribute to elevated levels of Survivin expression [147], which correlates with tumor aggressiveness [149] and resistance

to hormonal and chemotherapy [150, 151]. Studies conducted by Nastuik and colleagues have found that the FLICE-like inhibitory protein (FLIP), which is downregulated in the prostate during castration-induced apoptosis, is downregulated by TGF-β in NRP-152 and DU145 cells [152, 153]. Importantly, overexpression of FLIP protects against such apoptosis. These results suggest that TGF-β-induced death of prostate epithelial cells occurs also through an extrinsic pathway of apoptosis involving a death receptor and caspase-8.

Evidence Supporting Role of TGF-β as a Tumor Suppressor of Prostate

Analysis of TGF-β expression showed higher immunostaining for TGF-βs 1 and 2 in human prostate tumor epithelium relative to the surrounding stroma or their normal epithelial counterparts, while the intensity of TGF-β3 staining appeared similar but more apical in malignant versus a diffuse pattern in normal prostate epithelium [154]. However, immunohistochemical analysis of TGF-β receptors revealed a marked loss or reduction in the expression of TβRI, TβRII [155–158], and TβRIII [159–161] in prostate cancer, suggesting that TGF-β signaling responses are lost or reduced in malignant prostate tissues. Restoration of TGF-β receptor function by overexpression of TβRII was demonstrated to both enable growth suppression by TGF-β and greatly reduce the malignancy of the LNCaP human prostatic carcinoma cell line [162], inferring a tumor suppressor role of TGF-β receptor. The function of TGF-β as a tumor suppressor of prostatic epithelial cells was also tested by overexpression of a dominant-negative (DN)-TβRII in the NRP-152 nontumorigenic rat prostatic cell line. NRP-152 cells are unable to form tumors when implanted s.c. or under the renal capsule of athymic mice and have many properties of normal prostate epithelial cells, including AR expression, and high sensitivity to a variety of growth factors and TGF-βs [146]. Most unique about NRP-152 cells is their stem cell properties in vitro and in vivo [125, 163]. TGF-β promotes their growth arrest [146], apoptosis [131], and basal to luminal transdifferentiation [125]. DN-TβRII is a truncated form of TβRII that blocks TGF-β signaling by forming an inactive heteromeric complex with TβRI. Expression of DN-TβRII in NRP-152 cells inhibited their responses to TGF-β and triggered malignant transformation, as demonstrated by tumor growth in athymic mice [30]. Another nontumorigenic rat prostatic epithelial line named DP-153, developed from the dorsal prostate of a Lobund–Wistar rat also underwent malignant transformation upon expression of DN-TβRII [164].

The roles of Smads 2 and 3 as mediators of the tumor suppressor function of TGF-β were also investigated in NRP-152 cells. Smads 2, 3, and 2+3 were efficiently silenced in NRP-152 cells by lentiviral-mediated shRNA expression, and the silenced cell lines were implanted s.c. in athymic mice [148]. Unexpectedly, silencing Smad2 but not Smad3 alone caused malignant transformation, whereas silencing both Smads 2+3 caused an increase in the rate of tumor growth, suggesting that

Smad2 is the prominent mediator of tumor suppression by TGF-β in this model. The role of Smad2 is believed to be a property of the basal/stem cell-like phenotype of NRP-152 cells [148].

A number of studies using transgenic mice also support the hypothesis that TGF-β has potent tumor suppressor activity in the prostate. For example, targeted disruption of TGF-β signaling by expression of DN-TβRII in the prostates of transgenic mice, which was achieved by a C3 promoter, reduced levels of apoptosis and enhanced rates of epithelial cell proliferation in the prostate proximal ducts [121]. William and colleagues provided the first clear evidence that loss of TGF-β receptor signaling in prostate epithelium (by targeting DN-TβRII to the prostate with the zinc-inducible metallothionein (MT) promoter) increased metastatic progression of high-grade PIN lesions formed by SV40 large T antigen (Tag) targeted to the prostate with the large probasin promoter [165]. In a similar study, Pu and colleagues crossed the transgenic adenocarcinoma mouse prostate (TRAMP) mice with the MT-DN-TβRII mice and observed that dysregulation of TGF-β signaling in prostate tumors of TRAMP mice accelerated prostate tumorigenesis and metastatic progression [166]. Expression of DN-TβRII increased the rates of cell proliferation, apoptosis, and vascularity of TRAMP tumors. DN-TβRII induced an EMT response (loss of E-cadherin and β-catenin, and increased expression of N-cadherin and Snail) and increased levels of AR.

Further in vivo support for TGF-β's function as a tumor suppressor includes loss of TGF-β receptors in surrounding prostate fibroblasts, which strongly influences the growth and function of the epithelial compartment. Mice with targeted knockout of TβRII to fibroblasts developed invasive squamous cell carcinoma of the forestomach and prostate intraepithelial neoplasia [124].

The TGF-β Paradox

A prominent feature of TGF-β research has been the extreme and often contradictory functions of TGF-β in certain cancers, including PCa [88, 89]. Compelling evidence supports TGF-βs function as both tumor suppressor and tumor promoter in certain tissues. What is not entirely clear is how can TGF-β do this on the same tumors or cells. Is there a particular molecular switch or a discrete set of molecular switches during carcinogenesis that converts the function of TGF-β from a tumor suppressor to a tumor promoter? What is the normal physiological significance or evolutionary benefit of endowing a single protein with such potentially opposing functions? Accumulating evidence now supports the argument that although TGF-β functions as a tumor suppressor in the normal epithelium, it induces genes that are involved in growth suppression as well as in EMT, cell motility, and invasiveness. However, the tumor suppressor functions of TGF-β predominate in normal and premalignant tissues. Genetic alterations in cancer that ablate an upstream step in TGF-β receptor signaling, such as a frame-shift mutation in TβRII [167], may decapitate the pathway, thus promoting tumor growth [168]. However, in tumors

that exhibit an alteration in a downstream component of the TGF-β signaling pathway that ablates or suppresses, TGF-β-induced growth suppression and/or apoptosis tends to promote TGF-β's oncogenic effect. Mutations that decapitate TGF-β receptor signaling are rare in prostate cancer, enabling TGF-β's function as a tumor promoter in this gland.

Deregulation of TGF-β Signaling in PCa

In the previous sections we reviewed evidence from a number of studies that TGF-β functions as a tumor suppressor of the prostate, and that such tumor suppression is lost during the development and progression of PCa. This section highlights our current understanding of potential mechanisms by which TGF-β signaling is deregulated in PCa.

Alterations in AR Signaling on TGF-β Responses in PCa

AR expression and a dependence on androgens are retained in the majority of clinically localized or early-stage metastatic PCa. This has laid the foundation for androgen ablation as an important therapeutic option for surgically unresectable metastatic PCa [169]. While the initial response is generally very high, patients almost invariably relapse by 18–20 months with renewed tumor growth and rising serum PSA. Although the molecular basis for this relapse is not fully understood, accumulating evidence suggests that AR plays an important role in such relapse [128]. AR is a 110-kDa nuclear receptor that functions as a transcription factor to regulate the expression of numerous androgen target genes. Androgens promote the dimerization of AR [170], its nuclear translocation, and subsequent binding of AR to androgen response elements (ARE). AR-mediated transcription is regulated by numerous AR-associated proteins (ARAs) [171–176], along with CREB [177], RIP-140 [178], Ets [179], GRIP1 [180], F-SRC-1 [178], AP-1 [181], and Smads [134–137, 182].

Typically AR is amplified or overexpressed in PCa relative to normal prostate epithelium, and alterations in the expression of AR-binding proteins enhance responses of AR, leading to increase sensitivity to castrate levels of androgens (produced either by the adrenal gland or by PCa cells) [128]. The increased activation of AR through these mechanisms is likely to alter TGF-β signaling, as described earlier. To recap, androgens can downregulate the expression of all three TGF-β isoforms, along with TβRI and TβRII. Moreover, we showed that AR can repress the transcription of Smad3 (but not Smads 2 or 4) and TβRII. In fact, all AR-positive PCa cell lines studied have very low to undetectable levels of Smad3 and are deficient in TβRII, while the AR-negative cells are proficient in those TGF-β-signaling components. Transcriptional repression of Smad3 may account for the relatively low levels of Smad3 found in AR-positive PCa cells versus AR-negative cells or normal prostate epithelium [139, 183]. Expression profiling data of 102 human

prostate specimens (normal, tumor, hormone refractory) revealed an inverse correlation of the expression of AR with Smad3 ($p < 0.0001$) [139]. This suggests that the constitutive activation of AR signaling in CRPC is likely to disrupt key components in the tumor suppressive pathway of TGF-β.

While constitutive activation of AR may explain part of the mechanism by which TGF-β signaling is altered in PCa, other alterations in Smad3 may occur during prostate carcinogenesis, such as by activation of PI3K/Akt/mTOR signaling [184]. Importantly, Smad3 physically associates with AR, thereby repressing the transcriptional activity of AR [135] and likely the ability of AR to induce cell growth/survival [185]. Intriguingly, Smad3 heterozygote null mice have castrate-levels of circulating testosterone [186], yet have a normal-sized prostate, supporting the notion that loss of Smad3 sensitizes the prostate to castrate levels of androgens. In fact, deregulation of TGF-β signaling by DN-TβRII in TRAMP tumors enhanced the expression of AR [166]. Taken together, these results suggest that repression of TGF-β signaling or loss of Smad3 activity during PCa progression is likely to play a key role in enhancing the activation of AR and hence the progression to full-blown CRPC.

Impact of IGF-I/PI3K/Akt/mTOR Pathway on TGF-β Signaling

In conjunction with the overexpression or constitutive activation of AR, up to 50 % of prostate cancers exhibit constitutively activate PI3K/Akt/mTOR signaling [187]. In normal cells, activation of this pathway is typically triggered by tissue injury, inflammation, and repair processes. Cells respond to activated Akt by increasing survival and cell cycle progression, and they respond to activated mTOR by increasing protein synthesis and undergoing metabolic changes that ultimately help meet the demands of the regenerative/repair processes they confront [188]. Multiple mechanisms have been proposed for deregulation of this pathway in various cancers, including PCa, together providing strong incentive for the development of a vast arena of therapeutic drugs, some of which are currently under clinical investigation [187].

Of particular interest to PCa has been the insulin growth factor-I receptor (IGF-I, a 70 amino acid mitogenic peptide), which plays key roles in promoting the growth and survival of epithelial cells and is intimately tied into the control of carcinogenesis [25, 189–193]. Early studies showed a remarkably strong and positive correlation in the levels of serum IGF-I and the incidence of PCa in humans and implicated high serum levels of circulating IGF-I as an early predictor of PCa [194, 195]. Remarkably, enforced overexpression of IGF-I in transgenic mice promotes prostate carcinogenesis [196], suggesting a causal relationship between elevated IGF-I and the initiation or promotion of PCa. Activation of IGF-I signaling has also been proposed to be crucial to the development of an array of other carcinomas [197–200]. Although known mainly as a mitogen, the tumor-promoting activity of IGF-I is more likely through its function as an apoptosis inhibitor, shown to be mediated predominantly through Akt, also known as protein kinase B (PKB, a 57 kDa serine/

threonine kinase with an essential pleckstrin homology (PH)-domain). Activation of the IGF-I receptor (IGF-IR, a heterotetrameric transmembrane glycoprotein) by IGF-I, promotes receptor dimerization and autophosphorylation, generating phospho-tyrosines that enable the docking of class IA PI3-kinase (PI3K: consisting of 85 kDa regulatory subunit [p85α] and 100 kDa catalytic subunit [p110α]) through a src homology 2 (SH2). The recruited PI3K complex then catalyzes the conversion of phosphatidylinositol-4-phosphate (PtdIns{4}P) and PtdIns{4,5}P2 to PtdIns {3,4}P and PtdIns{3,4,5}P3, respectively, which localizes to the plasma membrane to provide an anchor for both Akt and the Akt kinase, PDK1, respectively [201]. PDK1 activates Akt by phosphorylating Akt at threonine 308. Akt is further activated for catalyzing a select group of targets through its phosphorylation on Serine 473 by PDK2, which is the mammalian target of rapamycin complex 2 (mTORC2). Not surprisingly, Akt is constitutively activated in >50 % of PCa and appears to be a reliable prognostic marker of such carcinomas [201–205]. The elevated activation Akt in PCa occurs through multiple mechanisms, principally through activation of receptor tyrosine kinases, and by functional inactivation of the tumor suppressor *p*hosphatase and *t*ensin homologue deleted on chromosome t*en* (PTEN) (found in 25–50 % of patients with PCa) [202, 206–211]. PTEN is a lipid phosphatase that keeps Akt activity in check by lowering the levels of PtdIns{3,4}P and PtdIns{3,4,5} P3 [212].

Data from our laboratory suggest that the IGF-I receptor promotes the survival of prostate epithelial cells, predominantly through reversing the ability of TGF-β to induce apoptosis [131]. Although IGF-I blocks apoptosis induced by a variety of stimuli through inactivation of late signals such as inactivation of Bad, caspase-9, and FOXO [213], we show that IGF-I also functions through an Akt-dependent mechanism to inhibit transcriptional responses by TGF-β in prostate epithelial cells, at least partly through intercepting the ability of TGF-β receptors to activate Smad3 [214]. This effect occurs through the activation of Akt and requires the kinase activity of Akt. Other results suggest that IGF-I's effects on suppressing Smad3 activation are at least partly through mTOR [184]. Interestingly, FKBP12, which is required for suppression of mTOR, also associates with TβRI and has been proposed to restrain ligand-independent activation of TβRI by TβRII [62, 215, 216]. However, rapamycin also reverses the ability of IGF-I to block the C-terminal phosphorylation of Smad3 by a constitutively active mutant form of TβRI that is defective for binding to FKBP12 [184]. This suggests that IGF-I suppresses Smad3 activation through an mTORC1-dependent mechanism. Akt can also directly associate with Smad3 and Smad2 [184] and prevent transcriptional responses of Smads in prostate epithelial cells and other cell types [184, 217].

Consistent with a tumor suppressor function of PTEN, which is lost in a high proportion of PCa, $PTEN^{p-/-}$ mice (targeted to the prostate with probasin-cre) develop adenocarcinoma after a long latency period with minimally invasive features [218]. Thus, metastatic progression of PCa must require other gene alterations in addition to PTEN. Gene expression profiling of $PTEN^{p-/-}$ prostate tumors from PTEN-null versus normal matched control prostate epithelium revealed strong activation of the TGF-β/BMP–SMAD4 signaling axis [219]. This contrasts with the

loss of Smad expression and activation found in advanced human PCa. Thus, to test the role of the activation of TGF-β/BMP in PTEN$^{p-/-}$ prostate tumors, Ding et al. [219] crossed PTEN$^{p-/-}$ with Smad4$^{p-/-}$ mice (which do not form prostate tumors). Resulting progeny developed metastatic and lethal prostate adenocarcinomas with 100 % penetrance. Pathological and transcriptomic knowledge-based pathway profiling analyses unveiled cell proliferation and invasion as two major features of these tumors. This also disclosed cyclin D1 and SPP1 as key co-mediators of these biological processes, together giving a four-gene signature marker list prognostic of aggressive PCa [219].

Other Mechanisms Likely Contribute to the Loss of Tumor Suppression by TGF-β in Prostate

Methylation of TβRI and TβRII promoters reported in a significant number of PCa clinical specimens showing loss of TGF-β receptor expression, and promoter demethylation studies in LNCaP cells suggest that such promoter methylation contributes to loss of TGF-β responses in prostate cancer [220]. ELAC2 is a prostate cancer tumor susceptibility gene product, which by virtue of physically binding to Smad2 mediates growth arrest by TGF-β in prostate cells [221]. Thus, functional loss of ELAC2 may contribute to loss of tumor suppression by TGF-β. PML is another tumor suppressor whose functional inactivation in PCa enhances activation of Akt by PTEN loss [222] and antagonizes Smad2/3-dependent TGF-β signaling [223]. The oncoprotein Ski physically associates with Smads 2, 3, and 4 at SBEs, thereby blocking TGF-β-induced growth arrest of prostate epithelial cells [224]. Ski is overexpressed in prostate cancer, likely contributing to loss of tumor suppression by TGF-β [225].

Function of TGF-β as a Tumor Promoter of the Prostate

The function of TGF-β as a potent immunosuppressive cytokine is commonly reported to impact on tumor escape from immunosurveillance and provides one of the mechanisms by which TGF-β can promote cancer progression [226]. Zhang et al. [227, 228] primed C57BL/6 mice with irradiated TRAMP-C2, and isolated their CD8+ T cells, which were subjected to retroviral transduction of either DN-TβRII or control vector and then transplanted into recipient mice challenged with a single injection of TRAMP-C2 cells. The group receiving adoptive transfer of TRAMP-C2-primed TGF-β-insensitive CD8+ T cells showed significant reduction or elimination of pulmonary metastasis. Immunofluorescence revealed that only TGF-β-insensitive CD8+ T cells permeated the tumors. In a similar study, adoptive transfer of TGF-β-defected TRAMP-C2 primed dendritic cells induced a potent tumor-specific cytotoxic T-cell response against TRMPC-C2 tumors [229].

These results support TGF-β's role in immunosurveillance of PCa and suggest that the increased expression of TGF-β in prostate carcinomas aids in tumor progression through this mechanism.

The above results support a paracrine role for TGF-β to function as a tumor promoter. However, accumulating evidence suggests that TGF-β can promote tumor progression through an autocrine mechanism involving TGF-β receptor signaling in the tumor parenchyma. Using the highly metastasis human PCa cell line, PC-3MM2, Zhang et al. [230] showed that suppression of TGF-β signaling in those cells by transfection with DN-TβRII significantly reduces the growth rate and metastatic incidence PC-3MM2 cells. Relative to control vector transfection, tumors with DN-TβRII had fewer blood vessels and reduced levels of IL-8. A study analyzing comparative effects of TGF-β on nontumorigenic human prostatic epithelial cell line BPH1 and three tumorigenic derivative sublines of BPH1 supports a role of TGF-β in the induction of EMT [231]. While the nontumorigenic BPH1 were growth inhibited by TGF-β, the three tumorigenic sublines were not only resistant to growth suppression by TGF-β but also underwent EMT in response to TGF-β. The authors provided evidence that high levels of phosphorylated Akt, which blocks Smad3 activation and p21 nuclear translocation, allow the tumorigenic sublines to escape cell cycle arrest and the activation of TGF-β-induced EMT and invasion. Prostate carcinoma-associated fibroblasts (CAF), which promote the malignant transformation of BPH1, may provide mechanistic insight into alterations that trigger the action of TGF-β as a tumor promoter. Relative to normal prostate fibroblasts, CAF express high levels of both TGF-β1, which was found to be necessary for the malignant transformation of BPH1 to CAF. CAF also produce high levels of stromal cell-derived factor-1 (SDF-1/CXCL12), which binds to CXCR4 on BPH1 cells [232]. Such malignant transformation of BPH1 by CAF was shown to be dependent on TGF-β-induced CXCR4 expression in BPH1, SDF-1, and perhaps other factors in CAF-conditioned medium, which together also activated the Akt signaling pathway and hence TGF-β-induced EMT.

Using a mouse model of progressive PCa, knockout of both PTEN and *p53* (two of the most common copy number deletions found in PCa), Yen-Nien and colleagues [233] showed a role for the transcription factors KLF4 and Slug in TGF-β-induced EMT. They derived a panel of clonal epithelial cell lines from probasin-Cre *Pten*$^{fl/fl}$; *Tp53*$^{fl/fl}$ tumors. One of these cell lines (AC3) that is capable of undergoing TGF-β-induced EMT was used to define the regulation of EMT and mesenchymal commitment. Slug, but not the other common mesenchymal marker Snail, was shown to be an initiator of TGF-β-induced EMT in vitro and in vivo. The induced expression of Slug was shown to require TGF-β-mediated degradation of KLF4 and also required loss of FOXA1.

Pin1, which is a peptidyl–prolyl cis/trans isomerase elevated in PCa, is critical for TGF-β-induced EMT in PC3 cells [234]. Moreover, elevated expression of Pin1 may suppress tumor suppression by TGF-β through proteosomal degradation of PML [223, 235]. Additional pathways shown to promote TGF-β induced EMT in PCa cells include c-Myc and EGF receptor signaling leading to activation of the

Ras–Raf–MEK1–Erk2 pathway [236]. The latter kinase causes Pin1-dependent degradation of PML and phosphorylates the Smad3 linker [235], which is critical to Pin1's interaction with Smad3 [234]. Phosphorylation of the linker region of R-Smads by other kinases elevated in PCa have been proposed to play a key role in the oncogenic function of TGF-β [82].

Another protein shown to play a role in TGF-β-induced EMT is the LIM protein ARA55/Hic-5, which is both an AR-binding and a TGF-β-inducible protein [237]. Hic-5 is expressed in PC3 and DU145 cells, and binds to Smad3 but not Smad2, thereby inhibiting certain Smad3-dependent transcriptional responses such as apoptosis [237]. However, in the same cells Hic-5 binds to Smad7, causing degradation of this inhibitory Smad and leads to preferential transcriptional activation by Smad2 over Smad3 [238]. Overexpression of AR in PC3 cells promotes DHT-induced loss of Hic-5 expression [239, 240] and suppression of EMT [241].

Plasma TGF-β as a Prognostic Marker of PCa

Numerous studies show that plasma levels of TGF-β1 are significantly elevated in patients with PCa. The first pilot study ($n=68$) demonstrated a statistically significant twofold elevation of plasma TGF-β1 in men with primary, stage II localized PCa over those with no cancer [242]. In contrast to elevation of PSA levels in men with BPH, plasma levels of TGF-β1 were not elevated in men with BPH relative to men without prostatic disease. Patients who presented with primary stage III/IV disease showed a statistically significant >2-fold increase in TGF-β1 levels relative to stage II patients, who showed no significant change in PSA. However, the levels of TGF-β in stage III/IV patients dropped only ~2-fold after prostatectomy, in contrast to a drop in PSA levels to base-line. A major challenge behind measurement of plasma TGF-β1 has been its preparation, since small amounts of platelet breakdown which is a rich source of TGF-β1 will lead to inaccurately high levels of plasma TGF-β1. Case in point, Wolff et al. [243] showed no change in the levels of TGF-β1 in serum of patients with BPH versus those with PCa. However, this suggests that the content of TGF-β1 in platelets of men with PCa is not elevated and that the elevated plasma TGF-β was tumor derived. Levels of IL-6 and plasma TGF-β1 but not serum GM-CSF and TNF-α were significantly elevated in patients with clinically evident metastases, correlating with levels of PSA [244]. In another study, plasma levels of TGF-β1 were demonstrated to be markedly elevated in men with PCa that metastasize to regional lymph nodes and bone [245]. The authors concluded that the preoperative level of plasma TGF-β1 in men without evidence of metastasis is a strong predictor of biochemical progression after surgery. Other clinical studies confirm those results [246–249] and provide strong rationale for using plasma TGF-β1 as a prognostic marker to help distinguish indolent from aggressive PCa.

TGF-β in PCa Therapeutics

Evidence presented in this chapter suggest that in advanced PCa, downstream TGF-β responses leading to the induction of apoptosis and growth arrest are repressed, whereas TGF-β responses promoting EMT, cell motility, invasion, angiogenesis, and evasion from tumor immune surveillance are enhanced. Multiple therapeutic strategies are currently in preclinical and early clinical stage for blockade of TGF-β or the oncogenic function of TGF-β signaling, falling into four distinct groups: (1) ligand traps, (2) antisense oligonucleotides (ASO), (3) small molecule kinase inhibitors, and (4) peptide aptamers [250]. Each approach has unique strengths, yet many challenges, and together offer a diverse spectrum of arsenal against various cancers. They necessitate prudent use to curtail the arrest of tumor suppression by TGF-β in normal tissues.

Ligand traps include TGF-β neutralizing antibodies, as well as TβRII [88, 251] and TβRIII-fused Fc portion of human IgG to increase stability [252]; they can be administered systemically or intratumorally. While Fc-TβRII binds to TGF-βs 1 and 3 but not TGF-β2, Fc-TβRIII effectively traps not only all TGF-β isoforms but also other members of the TGF-β superfamily. Administration of either Fc-TβRII or Fc-TβRIII showed good efficacy against metastastic tumor spread in transgenic mice [252, 253]. Oncolytic adenoviral administration of Fc-TβRII intratumorally or systemically reduced metastatic growth of human breast and PCa cells in athymic mice [254–256]. Tumor immune surveillance studies discussed earlier suggest that suppression of TGF-β signaling in tumor-reactive CD8+ T cells by ex vivo viral transduction of DN-TβRII could aid in the development of a prostate cancer vaccine.

All three TGF-β isoforms can be selectively trapped by the mouse monoclonal antibody 1D11, which effectively repressed growth of the metastatic 4T1 breast tumors in mice by inducing apoptosis through reducing the levels of IL-17 [257] and rescued bone loss due to osteolytic bone metastasis of the MDA-MB-231 human breast cancer line. TGF-β neutralizing humanized monoclonal antibody (GC-1008) is available for clinical use [258, 259] and currently in clinical trials for advanced renal cell carcinoma (RCC) and malignant melanoma [250]. GC-1008 is well tolerated and provides significant tumor responses including shrinkage of metastases [260].

A TGF-β2 ASO called AP12009 has been used to effectively treat glioblastoma and pancreatic cancer [261]. A Phase I/II high-grade glioma trial shows that AP12009 is well tolerated and offers significant survival benefit over standard chemotherapy [261]. AP12009 is currently under clinical investigation for treatment of advanced melanoma and colon cancer [250].

Numerous TGF-β receptor kinase inhibitors (SB-431542, Ki26894, SD-208, LY364947, SB-505124, LY2109761, LY2157299; Fig. 7.5) have been developed for preclinical and clinical use, with major advantages being their low production cost and the practicality of administration. The major disadvantages are their relatively low specificity and instability. Ongoing medicinal chemistry efforts seek to

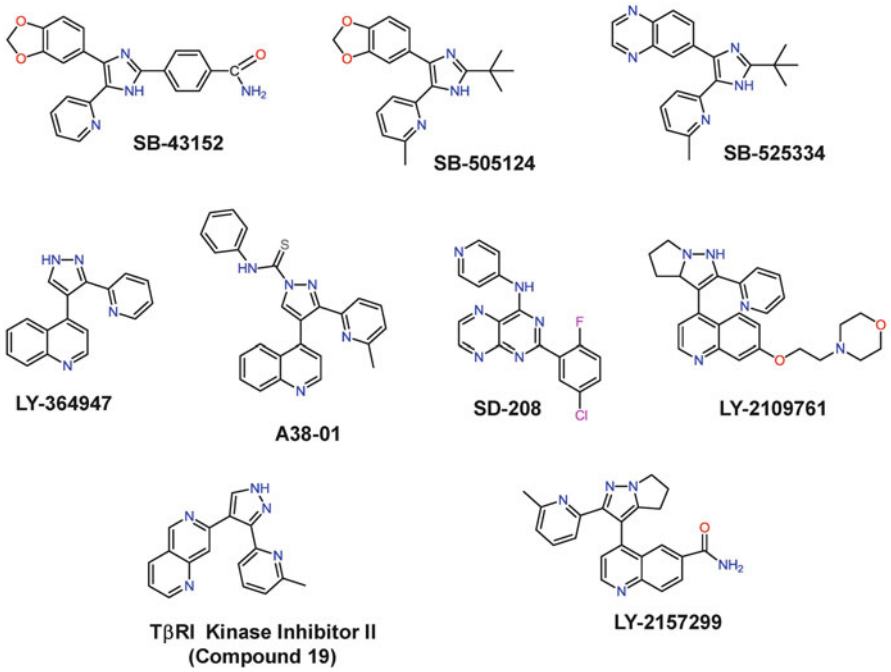

Fig. 7.5 Molecular structures of a number of selective small-molecule TβRI kinase inhibitors in preclinical and clinical use. Structures were compiled from various publications and drawn using ChemBioDraw Ultra 12.0 software

minimize those deficiencies. SB-431542 is generally used for cell culture studies, but is unstable in vivo [262]. Ki26894 and LY364947 have been demonstrated to be effective in reducing tumor metastasis and prolonging survival in mice [263, 264]. Oral administration of SD-208 prevented the development of osteolytic bone metastases with a significant reduction in size of osteolytic lesions after 4 weeks in mice [265]. LY2109761 has shown good efficacy in inhibiting metastatic spread of a variety of cancers including PCa to bone in mouse models [266–268] and resulted in less bone loss by PCa-induced osteoclasts [269]. Suppression of TGF-β in the spread of PCa to the bone appears to be critical to prevent a viscous cycle of TGF-β signaling through the release and activation of both TGF-β and BMPs that are enriched in bone. While SB-505124 is recommended for high specificity [270], LY2157299 showed rather favorable pharmacokinetics and low toxicity in a Phase I trial and is now entering a Phase II trial for various metastatic malignancies [250].

Small peptide aptamers offer the potential to selectively target an oncogenic arm of TGF-β signaling, leaving the tumor suppressive arm intact. Trx-SARA is an aptamer that inhibited TGF-β-induced EMT in NMuMG murine mammary epithelial cells in vitro by disrupting interactions of Smads 2 and 3 with Smad4 [271]. Underutilized alternative strategies for blocking TGF-β signaling may involve siRNAs for silencing of TβRI, TβRII, or Smads 2, 3, or 4. Future efforts are likely to

involve innovative strategies tailored to more precisely target the tumor microenvironment to defeat the oncogenic arm of TGF-β while preserving its tumor suppressing function.

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Chapter 8
The p38 MAPK Pathway in Prostate Cancer

Daniel Djakiew

Abstract The conventional signal transduction pathway for p38 MAPK is complex and diverse. A plethora of signals such as growth factors interact with death receptors to initiate a biochemical cascade by recruitment of activator molecules that in combination activate MAP3Ks. Many drugs intercede at the level of signal, activator, or MAP3Ks to mimic initiation of the signal transduction cascade. In the prostate, these signaling moieties, which include NSAIDs, converge at the level of MAP2Ks, ostensibly MKK6, which phosphorylates up to 4 isoforms of p38 MAPK. Phosphatases such as MKP1 or compounds such as biochanin A are able to antagonize activation of p38 MAPK. Phosphorylation of p38 MAPK allows phosphorylation of MK2 and MK3 that in turn promote stability of the $p75^{NTR}$ transcript. Concurrently, translocation of HuR from the nucleus to the cytoplasm and increased levels of HuR and eIF4E also promote $p75^{NTR}$ mRNA stability and increased levels of the $p75^{NTR}$ protein. In the prostate, the $p75^{NTR}$ functions as both a tumor and metastasis suppressor. In this context, increased expression of $p75^{NTR}$ modulates cell cycle effectors producing cytostasis in G0/G1, as well as mitochondrial effectors that modulate a caspase cascade leading to apoptosis. In addition, increased expression of $p75^{NTR}$ modulates motility effectors, ostensibly NAG-1, that retards cell migration. Hence, activation of the p38 MAPK pathway through a plethora of signal initiating events, leads to tumor and metastasis suppressor activity in prostate cancer cells.

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The Conventional p38 MAPK Pathway

Two distinct classes of stress-activated protein kinases (SAPK) have been identified in mammals, consisting of the p38 mitogen-activated protein kinases (MAPKs) and c-Jun N-terminal kinases (JNKs) of which the later exhibits limited cross-talk with the p38 MAPKs. There are four mammalian genes that encode p38 MAPKs. MAPK14 encodes p38α, MAPK11 encodes p38β, MAPK12 encodes p38γ, and MAPK13 encodes p38δ. Several alternatively spliced forms [1–3] produce at least ten isoforms of the p38 MAPK family. p38α and p38β are ubiquitously expressed; whereas p38γ is found predominantly in skeletal muscle and p38δ is enriched in lung, kidney, pancreas, small intestine, and T cells [4]. All the p38 MAPKs are characterized by the presence of a conserved Thr–Gly–Tyr (TGY) phosphorylation motif in their activation loop [5]. Although all p38s share a similar mechanism of action and substrate specificity, their distinct sequence identity and differential sensitivity to inhibitors suggest that they can be subdivided into two groups, p38α + p38β and p38γ + p38δ [6]. Within the first group, p38α has been the most widely studied isoform and tends to be expressed at higher levels than the p38β isoform.

The signaling pathway for the p38 MAPK is complex and diverse. Nevertheless, the conventional pathway can be summarized as usually being initiated by a **signal** (e.g., death receptor), coupled to an intracellular **activator** (e.g., TRAF2) that interacts with a **MAP3K** (e.g., ASK1), that phosphorylates a **MAP2K** (e.g., MKK6) that subsequently phosphorylates **p38 MAPKs** initiating interactions with **substrates** that lead to responses such as differentiation, senescence, and apoptosis (Fig. 8.1) consistent with tumor suppressor activities [7]. Although some studies have reported prosurvival functions of p38α, the vast majority of studies associate p38α activity with induction of apoptosis by cellular stresses [8]. Environmental stresses such as osmotic shock, chemotherapeutic agents [9], heat, and UV light [10] may also initiate signal transduction of the p38 MAPK pathway via receptor-independent mechanisms that induce changes in membrane fluidity or other specialized signaling systems [11]. Extracellular proinflammatory cytokines and growth factor stimuli that regulate the p38 MAPK pathway include nerve growth factor, GM-CSF, fibroblast growth factor, insulin-like growth factor 1, and platelet-derived growth factor [12]. Cytokines such as Il-1β, IL-6, and TNF-α [13, 14] are also well documented to control p38 MAPK-dependent pathogenesis of inflammatory responses [15]. Signals such as TNF-α and IL-1 activate TRAF adaptor proteins by recruitment to the intracellular domains of their cognate receptors [16]. The p38 isoforms are also activated by GPCRs [17] and the Rho family GTPases, Rac and Cdc42 [18]. Activator proteins then promote MAP3K activity. Several MAP3Ks (MEKK1-4, MLK, ASK1, YAO, TAK1) mediate intermediate signals from upstream activators (Cdc42, TRAF2, GPCRs, Rac). Moreover, thioredoxin (Trx) sensing of reactive oxygen species (ROS) appears to facilitate TRAF2 binding and activation of apoptosis signal-regulating kinase 1 (ASK1) [19], which promotes p38 MAPK-dependent apoptosis [20]. The plethora of signals, activators, and MAPK3 converge on two

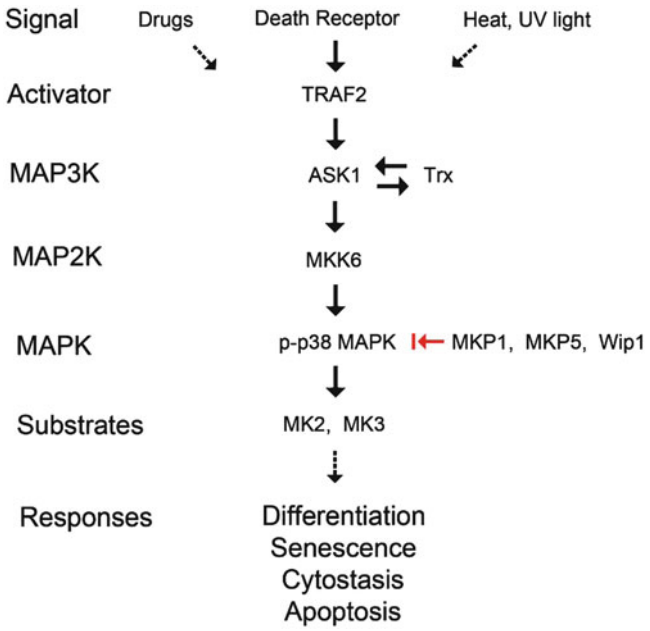

Fig. 8.1 The conventional signal transduction pathway that converges at the p38 MAPK is initiated by a signal (e.g., death receptor), coupled to an intracellular activator (e.g., TRAF2) that interacts with a MAP3K (e.g., ASK1), that phosphorylates a MAP2K (e.g., MKK6) that subsequently phosphorylates p38 MAPKs initiating interactions with substrates that lead to responses such as differentiation, senescence, and apoptosis. Solid arrows represent direct interactions whereas dashed arrows represent indirect interactions. The *red arrow* represents a block to activity

predominant MAP2Ks, MMK3 and MMK6. MKK6 activates all four isoforms of p38; whereas MKK3 activates p38α, p38γ, and p38δ, consistent with suggestions that MKK6 is the major activator of p38 MAPK [21]. MKK4, better known to activate the JNKs, has also been shown to phosphorylate p38α and p38δ under select conditions [22].

Signal transduction in a variety of tissues that regulates p38 MAPK phosphorylation is also mediated by phosphatases that control duration of signaling (Fig. 8.1). Phosphatases with growth promoting (oncogenic) activity that target p38 MAPK include MKP-1 [23], MKP-5 [24], Wip1 [25], and HePTP/LC-PTP [26]. Phosphatases with growth inhibitory (tumor suppressor) activity that target p38 MAPK include MKP-4 [24], PAC-1 [27], and PP2Cα-2 [28]. The M3/6 and PTP-SL/STEP phosphatases are also known to target p38 MAPK with unknown activity.

Downstream targets of p38α MAPK include protein kinases MK2, MK3 with some crosstalk to MK5 (PRAK) [29]. MK2 and MK3 are activated under a number of stress conditions such as UV radiation, heat shock, oxidative stress, hyperosmolarity, and cytokines that first stimulate activation of p38 MAPK isoforms [30].

MK2 increases production of TNF-α and IL-6 by promoting stability of their mRNAs [31]. MK2 has also been shown to phosphorylate a number of proteins such as human R-antigen (HuR) that facilitate translation of effector proteins [32]. MK2 and MK3 also appear to regulate tissue-specific gene expression and cell differentiation [33] by binding to the polycomb group proteins [34, 35]. MK2 also regulates cell cycle by phosphorylating CDC25B and CDC25C in response to UV radiation [36].

A body of evidence has shown that the p38 MAPK pathway is associated with a number of cellular responses related to cancer. In some cases the findings are contradictory. However, the majority of studies now suggest that the p38 MAPK pathway promotes tumor suppressor activity in cancer cells (Fig. 8.1). In this context, inactivation or knockout of the p38 MAPK pathway enhances cell transformation [29, 37]. Conversely, activation of p38 MAPK has been shown to induce apoptosis by NGF withdrawal [38] and Fas ligation [39]. The p38 MAPK negatively regulates cell cycle progression [40] by downregulating cyclins and upregulating cyclin-dependent kinase inhibitors that retard progression through the G1/S and G2/M transitions [41, 42] to promote growth arrest [37], cellular senescence [43], and apoptosis [40]. Moreover, many chemotherapeutic agents require p38 MAPK activity for induction of apoptosis [9, 44].

The general pathway for p38 MAPK activation and cellular response (Fig. 8.1) involves a plethora of signals, activators, MAP3Ks, MAP2Ks, phosphatases, and substrates in diverse tissues and cells. Different combinations of these intermediaries provide redundancies of signal transduction. Moreover, parallel pathways may provide limited cross-talk that regulates overall activity. In prostate cancer, specific components of this pathway have been identified to regulate apoptosis.

The p38 MAPK Signal Transduction Pathway in the Prostate

In the human prostate the p38 MAPK proteins and its phosphorylated forms have been shown to be expressed in pathologic cancer tissues [45, 46]. In particular there appears to be a progressive increase in phosphorylated p38 MAPK in the epithelia of normal, BPH, and cancer cells [46]. Animal models of prostate cancer are generally confirmatory for the expression of p38 MAPK observed in the human pathology [47]. Upstream MAP2Ks that activate p38 MAPK, such as MKK6, are also overexpressed in human prostate cancer tissues [48]. Conversely, pathologic expression of the phosphatase MKP-1 that targets p38 MAPK, decrease with higher histologic grade of prostate cancer [23]. Hence, the pathologic expression of these proteins is consistent with an overall upregulated phosphorylation of p38 MAPK in prostate cancer.

At the molecular level a substantial body of evidence has shown that several of the death receptors are able to initiate p38 MAPK signal transduction. Many of these receptor proteins including the $p75^{NTR}$, $p55^{TNFR}$, Fas, death receptors 3–6, and EDAR have similar sequence motifs of defined elongated structure [49] designated "death domains" based upon their apoptosis inducing function [50]. In most cases receptor ligation initiates signal transduction with the exception of $p75^{NTR}$ where ligand withdrawal promotes apoptosis [51] in prostate cancer cell lines [52]. Several NSAID drugs have been shown to increase $p75^{NTR}$ expression levels [53–55] via p38 MAPK phosphorylation in prostate cells. In the absence of additional ligand upregulated expression of $p75^{NTR}$ favors a stoichiometry of un-liganded receptor leading to p38 MAPK-dependent apoptosis [53–55]. The target of these NSAIDs (ibuprofen, r-flurbiprofen, carprofen) in prostate cells remains to be established. However, both ibuprofen and r-flurbiprofen stimulate MKK6 phosphorylation but not ASK-1 phosphorylation within 30 s of treatment [56]. Similarly, a number of other drugs have been shown to stimulate p38 MAPK signal transduction upstream of the MAP2Ks (Fig. 8.1). Drugs that also induce p38 MAPK-dependent apoptosis include 2-methoxyestradiol [57], melatonin [58], proanthocyanidins [59], raloxifene [60], and protoapigenone [61]. Given the diversity of drugs that induce p38 MAPK-dependent apoptosis it seems likely that the target molecules of these drugs may also be diverse with the potential for cross-talk from parallel pathways.

Phosphorylation of p38 MAPK can be antagonized by the phosphatase MKP-1 [23] and the phytoestrogen-like flavonoids, genistein and biochanin A. Genistein has been shown to inhibit drug-induced phosphorylation of p38 MAPK in prostate cancer cells [62]. Biochanin A is even more potent than genistein in inhibiting drug-induced phosphorylation of p38 MAPK, leading to suppressed levels of the $p75^{NTR}$ protein and concomitant-enhanced cell survival [63].

Drug-induced activation of the p38 MAPK pathway leads to rapid phosphorylation of MK2 and MK3 within 5 min of treatment in prostate cancer cells. Subsequent siRNA knockdown of either MK2 and/or MK3 reduces drug-induced expression of $p75^{NTR}$ levels [54, 55]. Downstream of MK2/3 drug-induced activation of p38 MAPK is associated with translocation of HuR from the nucleus to the cytoplasm where HuR binds to the $p75^{NTR}$ transcript [54] promoting $p75^{NTR}$ mRNA stability [64] and enhanced protein expression levels [53]. Conversely, siRNA knockdown of HuR reduces drug-induced levels of the $p75^{NTR}$ protein [53]. In addition, drug-induced activation of the p38 MAPK pathway promotes increased phosphorylation of the translation initiation protein eIF4E [53]. Subsequent knockdown of p38α MAPK reduces drug-induced phosphorylation of eIF4E levels [53] in prostate cancer cells. Since both HuR and eIF4E are known to contribute to mRNA stability, it appears that certain NSAID drugs (ibuprofen, r-flurbiprofen, carprofen) induce phosphorylation of the p38 MAPK pathway that subsequently promotes stability of the $p75^{NTR}$ transcript allowing enhanced translation of $p75^{NTR}$ protein in prostate cancer cells (Fig. 8.2).

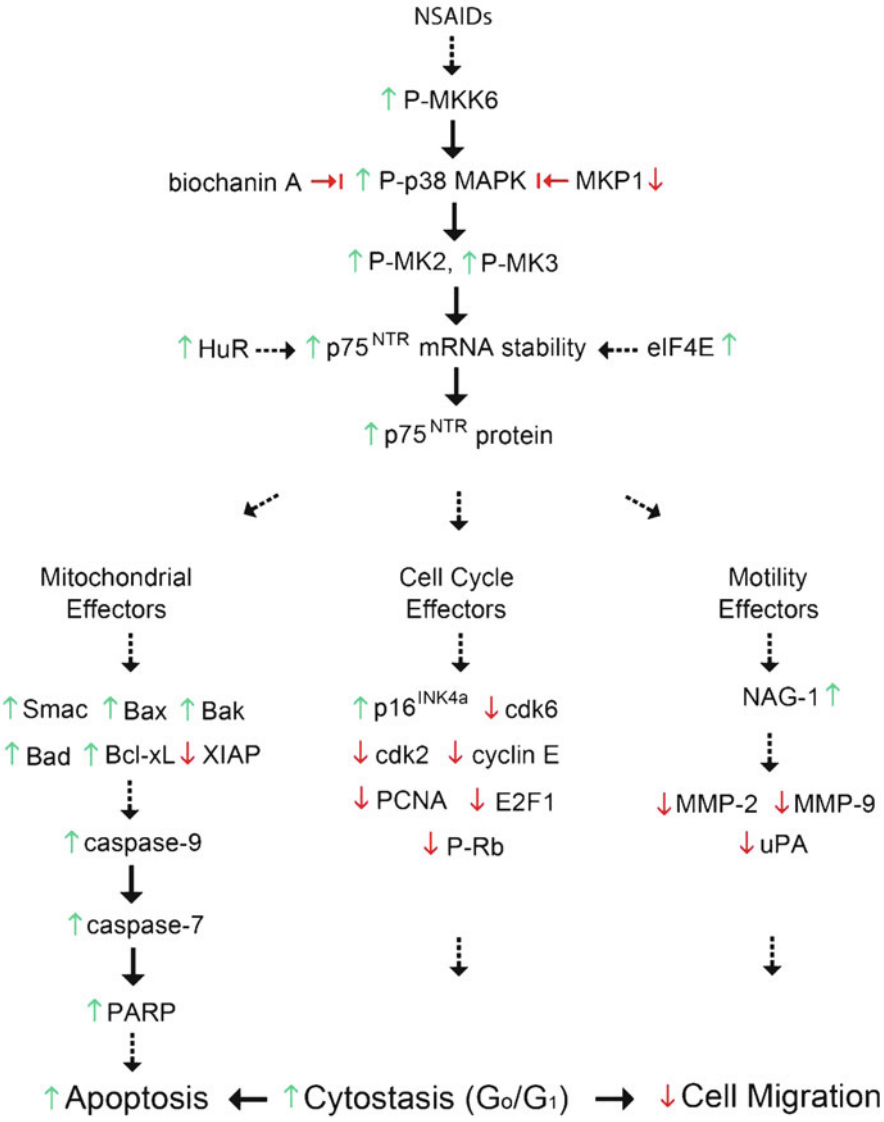

Fig. 8.2 In prostate tissues and tumor cells the signal transduction pathway can be initiated by a number of stimuli such as NSAIDs that lead to phosphorylation of MKK6. MKK6 phosphorylation of p38 MAPK can be antagonized by isoflavone compounds such as biochanin A and by phosphatases such as MKP1. Activated p38 MAPK then phosphorylates MK2 and MK3 that induces stability of the $p75^{NTR}$ transcript in the context of activated HuR and eIF4E leading to increased expression of the $p75^{NTR}$ protein. Increased expression of the $p75^{NTR}$ protein leads to modification of mitochondrial effectors of the intrinsic apoptotic pathway, cell cycle effectors, and motility effectors all of which promote cytostasis, apoptosis, and inhibition of cell migration. Solid arrows represent direct effects whereas *dashed arrows* represent indirect effects. *Green arrows* indicate increased expression whereas *red arrows* indicate reduced expression

Induction of the p38 MAPK Pathway Retards Cell Cycle Progression in Prostate Cancer Cells

Drug-induced phosphorylation of the p38 MAPK induces cytostasis [65] in the G0–G1 phase of the cell cycle in prostate cancer cells [66–68]. In part, this can be related to drug-induced expression of $p75^{NTR}$ via the p38 MAPK pathway [55] and/or genetic overexpression of $p75^{NTR}$ [52, 53] both of which promote cell cycle arrest [69] in prostate cancer cells. In addition, re-expression of $p75^{NTR}$ induces partial re-differentiation of prostate cancer cells by inducing increased levels of RAR-β, RXR-α, RXRβ, and CRABP1 [70] thereby sensitizing cells to 9-cis retinoic acid induced arrest in G1 [71]. Increased expression of $p75^{NTR}$ promotes cytostasis with concomitant alterations in the levels of the cyclin/cdk holoenzyme complex [52]. In this context, upregulated expression of $p75^{NTR}$ is associated with reduced levels of cdk6 and PCNA, and increased levels of $p16^{INK4a}$, which promote hypo-phosphorylation of Rb [52] associated with retarded progression through G1 of the cell cycle [72]. Conversely, the rescue of cdk6, PCNA and phosphorylated Rb levels, and suppression of $p16^{INK4a}$ levels by a death domain-deleted dominant-negative antagonist of $p75^{NTR}$ and alternatively by addition of NGF ligand allows increased progression through G1 thereby showing a $p75^{NTR}$-dependent regulation of G1 in prostate cancer cells [52]. Increased expression of $p75^{NTR}$ also promotes cytostasis by preventing cell cycle progression through the G1/S restriction point, ostensibly by suppressing levels of cyclin E, cdk2, and the E2F1 transcription factor that stabilizes hypo-phosphorylated Rb [52]. Normally hypo-phosphorylated Rb binds E2F1 so that the Rb/E2F1 complex can no longer promote transcription of PCNA thereby preventing progression into the S phase of the cell cycle [73]. Conversely, the rescue of cyclin E, cdk2, and E2F1 levels by a death domain-deleted dominant-negative antagonist of $p75^{NTR}$ and alternatively by addition of NGF ligand allows increased progression into the S phase thereby showing a $p75^{NTR}$-dependent regulation of the G1/S restriction point in prostate cancer cells [52]. Prolonged $p75^{NTR}$ dependent cytostasis, either through drug-induced p38 MAPK signal transduction or genetic upregulated expression of $p75^{NTR}$ appear to predispose these cancer cells to eventual apoptosis [52].

Induction of the p38 MAPK Pathway Promotes Apoptosis in Prostate Cancer Cells

Elevated expression of the $p75^{NTR}$ tumor suppressor protein via drug-induced activation of the p38 MAPK pathway [55] or genetic overexpression [69] leads to apoptosis of prostate cancer cells [52, 53, 55]. Rescue of drug-induced activation of p38 MAPK [52, 54]-dependent expression of $p75^{NTR}$ by siRNA knockdown [53], or a death domain-deleted dominant-negative antagonist of $p75^{NTR}$ [52–55], and alternatively by addition of NGF ligand [52] all enhance survival [53, 55] and all prevent

apoptosis [52, 53, 55]. The apoptosis inducing activity of $p75^{NTR}$ appears to occur through the mitochondrial intrinsic pathway [52]. Significantly, $p75^{NTR}$ is expressed in the inner mitochondrial membrane of a wide variety of tissues [74]. Elevated expression of $p75^{NTR}$ induces mitochondrial expression of pro-apoptotic Smac, Bax, Bak, and Bad and reduces expression of the pro-survival effector, Bcl-xL [52]. A death domain-deleted dominant-negative antagonist of $p75^{NTR}$ or alternatively addition of NGF ligand both reverses expression of these mitochondrial effectors [52]. Subsequent elevated expression of $p75^{NTR}$ induces a caspase-9 and caspase-7 cascade [52] by first suppressing levels of XIAP that otherwise binds and inhibits activation of caspase-9 and caspase-7 [75]. Elevated expression of $p75^{NTR}$ induces cleavage and activation of caspase-9 followed by caspase-7 leading to PARP cleavage and apoptosis [52]. A death domain-deleted dominant-negative antagonist of $p75^{NTR}$ or alternatively addition of NGF ligand rescues levels of XIAP, prevents cleavage of caspase-9, caspase-7, and PARP which in turn prevents apoptosis [52]. Hence, drug-induced activation of the p38 MAPK pathway leading to elevated $p75^{NTR}$ expression induces caspase-dependent apoptosis of prostate cancer cells.

Induction of the p38 MAPK Pathway Inhibits Cell Migration in Prostate Cancer Cells

NSAID-induced activation of p38 MAPK inhibits prostate cancer cell migration [76]. NSAID activation of p38 MAPK induces a signal transduction cascade leading to elevated expression of $p75^{NTR}$ and NSAID-activated gene-1 (NAG-1) downstream of $p75^{NTR}$ [76]. Expression of both $p75^{NTR}$ and NAG-1 retards cell migration [76]. Pharmacological inhibition of p38 MAPK activity, or siRNA knockdown of either p38α MAPK or p38β MAPK reduces expression of NAG-1, and siRNA knockdown of NSAID-induced NAG-1 rescues cell migration in prostate cancer cells [76]. Hence, NSAID-induced phosphorylation of p38 MAPK increases NAG-1 expression and concomitantly reduces cell migration (Fig. 8.2). Since NSAID-induced expression of $p75^{NTR}$ inhibits both cell survival and cell migration [76], it would appear that $p75^{NTR}$-dependent inhibition of migration may be a function of the reduced viability of these cells. In contrast, NAG-1 expression only inhibits cell migration, but not cell survival of prostate cancer cells, consistent with an archetypical metastasis suppressor activity independent of p38 MAPK-induced $p75^{NTR}$ metastasis suppressor activity [76]. Significantly, treatment of the DU-145 prostate cancer cell line with proanthocyanidins increases phosphorylation of p38 MAPK and levels of TIMP-2, while concurrently inhibiting MMP-2 and MMP-9 activity [77]. These results are also consistent with observations that increased expression of $p75^{NTR}$ inhibits activity of urokinase plasminogen activator, MMP-2 and MMP-9, while increasing levels of TIMP-1 [78]. A death domain-deleted dominant-negative antagonist of $p75^{NTR}$ reverses activity of uPA, MMP-2, MMP-9, and levels of TIMP-1 [78]. Many different drugs have been shown to both increase and decrease phosphorylation of p38 MAPK and increase or decrease protease activity, in many

cases with opposing results. However, observations that some select drugs (NSAIDs) consistently induce activity of p38 MAPK leading to increased levels of NAG-1 and upstream levels of $p75^{NTR}$, then $p75^{NTR}$-dependent inhibition of uPA, MMP-2, MMP-9, and increased TIMP-1 [78], support $p75^{NTR}$ associated metastasis suppressor activity of this pathway [69]. Hence, it appears that some drugs, such as NSAIDs, activate the p38 MAPK pathway leading to downstream expression of $p75^{NTR}$ and NAG-1, thereby inhibiting cell migration consistent with a metastasis suppressor phenotype (Fig. 8.2).

Summary and Conclusions

Activation of the p38 MAPK pathway in prostate epithelia is generally inductive of differentiation, cytostasis, reduced cell migration, and apoptosis (Fig. 8.2). A plethora of stimuli including growth factors, drugs, osmotic shock, and redox may contribute to a diverse set of initiating signals that at times produce seemingly conflicting biochemical cascades with different responses. Indeed, the different signals, activators, and MAP3Ks representative of the conventional pathway (Fig. 8.1) derived from many different cell lines and tissues suggest a network of disparate signal transduction components amenable to cross-talk stimuli that nevertheless appear to converge at the level of the MAP2Ks, which consistently appears to be MKK6 in the prostate (Fig. 8.2). Activation of p38 MAPK can regulate a wide range of substrates that facilitate mRNA stability and activation of transcription factors. In the prostate a number of studies support phosphorylation of p38 MAPK driving expression of the $p75^{NTR}$ suppressor protein that in turn inhibits survival through differentiation and cytostasis leading to apoptosis and reduced cell migration (Fig. 8.2). Hence, activation of the p38 MAPK pathway leads to tumor and metastasis suppressor activity in prostate cancer cells.

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Chapter 9
NF-kappaB2/p52 in Prostate Cancer

Nagalakshmi Nadiminty, Ramakumar Tummala, Yezi Zhu, and Allen C. Gao

Abstract The NF-κB transcription factor family members play pivotal roles in several biological and molecular processes. Two pathways of activation of NF-κB family members exist: a classical pathway involving a sequence of events leading to nuclear translocation of p65:p50 heterodimers and a non-canonical pathway involving the partial signal-induced processing of NF-κB2/p100 and the nuclear translocation of p52-containing heterodimers. NF-κB proteins are involved in a variety of lymphoid and epithelial cancers as well as prostate cancer. In this chapter, the role of classical NF-κB is briefly described while the novel role of NF-κB2/p52 in prostate cancer is discussed in detail. Activated STAT3 mediates enhanced processing of p100 leading to higher levels of p52 in prostate cancer. NF-κB2/p52 is overexpressed in prostate cancer, interacts with the androgen receptor in prostate cancer, and enhances ligand-independent growth and survival of prostate cancer cells. In addition, p52 regulates a wide variety of target genes that are involved in metastasis and angiogenesis. Downregulation of endogenous p52 in prostate cancer cells inhibits prostate cancer growth. NF-κB2/p52 is strongly implicated in the progression of castration-resistant prostate cancer, suggesting that targeting this transcription factor may have beneficial effects in therapy of this disease.

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Prostate Cancer

Prostate cancer (PCa) is the second most commonly diagnosed malignancy in men and a leading cause of cancer death in men in the USA [1]. Surgery and radiation are common primary treatment regimen for localized PCa. Recurrent disease is often treated with androgen deprivation therapy (ADT). However, most, if not all, patients eventually fail ADT and develop castration-resistant PCa (CRPC) which is often refractory to currently used therapeutic agents. At this stage, when treated with ADT, PCa progression occurs in the face of very low levels of circulating androgens. The androgen receptor (AR), a steroid hormone receptor, plays a major role in prostatic development, cellular transformation, and progression of both early and late stages of PCa. Unbound receptor is complexed with heat shock proteins and is retained in the cytoplasm, while ligand binding results in nuclear translocation and expression of many target genes such as *PSA* and *TMPRSS2*. From a developmental standpoint, AR is a key player in survival and maintenance of prostatic epithelial cells by regulating the secretion of paracrine growth factors from stromal cells [2]. During PCa initiation, the paracrine mechanism is substituted by an autocrine AR-dependent activation, which signals for undeterred growth, angiogenesis, and migration [3]. Ablation of testicular androgens in hormone-sensitive PCa leads to suppression of circulating androgen levels and downregulation of AR signaling, culminating in tumor regression [4]. Persistent aberrant reactivation of the AR after ADT is the hallmark of CRPC and occurs by a variety of mechanisms including AR gene amplification and overexpression [5], promiscuous ligand recognition [6], intracrine androgen synthesis [7], and ligand-independent AR activation either by gain-of-function mutations in AR [8] or emergence of constitutively active AR splice variants lacking the C-terminal region [9]. Past as well as recent clinical experience supports the role of reactivation of the AR in CRPC, and a major focus of current research is the identification of new agents that would prevent and/or delay the development of CRPC or slow its progression by targeting the expression/activation of the AR.

Several molecular pathways have been implicated in prostate cancer progression such as growth factor receptor-mediated signaling pathways and constitutive activation of transcription factors such as NF-κB and STAT3 [10]. Constitutive activation of NF-κB has been demonstrated in PCa, and patients displaying elevated nuclear levels of NF-κB have decreased overall survival rates [11]. NF-κB plays a major role in the control of immune responses and inflammation and promotes malignant behavior by increasing the transcription of the anti-apoptotic gene *BCL-2* [12], cell cycle progression factors such as *c-MYC* and *cyclin D1*, proteolytic enzymes such as *matrix metalloproteinase 9* (*MMP-9*) and *urokinase-type plasminogen activator*, and angiogenic factors such as *VEGF* and *IL-8* [13, 14]. NF-κB also provides an adaptive response to PCa cells against cytotoxicity induced by redox-active therapeutic agents and is implicated in radiation resistance of cancers [15, 16].

NF-κB Proteins

Nuclear Factor-kappa B (NF-κB) transcription factor family members play critical roles in cell survival, inflammation, immune response, and transformation controlling the expression of an exceptionally large number of genes [17]. This family is composed of structurally homologous transcription factors, namely RelA, RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52). They bind to and transactivate diverse structural elements in gene promoters as either homo or heterodimers. In most cells NF-κB homo and heterodimers are held latent in the cytoplasm in complex with inhibitors of κB (IκB) proteins. The IκB family includes IκBα, IκBβ, IκBγ, IκBε, Bcl-3, and the precursors of NF-κB1 and NF-κB2 (Fig. 9.1). NF-κB1 and NF-κB2 precursor proteins generate the active subunits p50 and p52 upon co- and post-translational processing respectively [18]. All members of the family contain an N-terminal Rel Homology Domain (RHD), which is responsible for DNA binding and dimerization. Rel A, Rel B and c-Rel also contain a C-terminal Transactivation Domain (TAD) while NF-κB1 and NF-κB2 lack a TAD. In addition, NF-κB1 and NF-κB2 proteins possess a C-terminal ankyrin repeat domain (ARD) and a death domain (DD), which function as processing inhibitory structures (Fig. 9.1). NF-κB1 is usually processed co-translationally to p50 [19], whereas NF-κB2 processing is

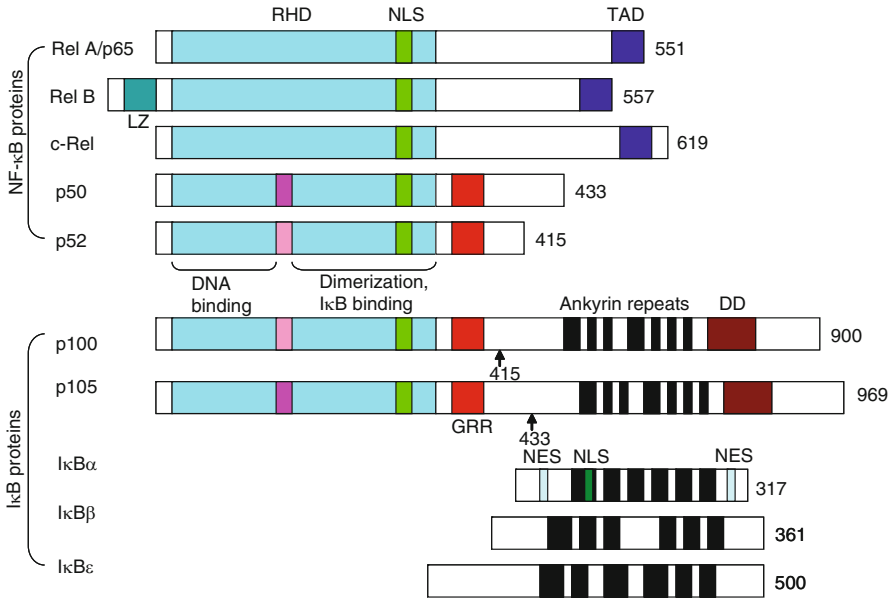

Fig. 9.1 Schematic representation of NF-κB and IκB proteins. *RHD* rel homology domain, *NLS* nuclear localization sequence, *NES* nuclear export sequence, *TAD* transactivation domain, *DD* death domain, *GRR* glycine-rich region

tightly regulated and is signal dependent in many cell types [20], and is required to prevent abnormal processing into p52, which exhibits oncogenic functions in a subset of lymphomas.

Classical NF-κB Pathway

Classical NF-κB is activated by a wide variety of stimuli, such as proinflammatory cytokines, bacteria, viruses, viral proteins, and T- and B-cell mitogens, thus activating a variety of cell surface receptors [20]. NF-κB proteins are retained in the cytoplasm by IκB proteins, characterized by 6–7 C-terminal ankyrin repeats that are required for binding to the RHD and to mask the NLS [21]. The best characterized of the IκB family members is IκBα. An inflammatory stimulus such as TNF-α, engages its receptor, leading to activation of the IKK complex and phosphorylation of IκBα at Ser 32 and Ser 36 [22]. The kinase complex responsible for the phosphorylation of IκB proteins consists of two highly homologous catalytic subunits IKK1 (IKKα) and IKK2 (IKKβ) and a regulatory subunit NEMO (IKKγ). IKKβ and IKKγ are required for the activation of the classical NF-κB in response to proinflammatory stimuli, whereas IKKα is dispensable. Phosphorylated IκBα is polyubiquitinated at Lys 19 by the Skp1, Cdc53/Cullin1, and F-box protein β-transducin repeat-containing protein (β-TrCP) $SCF^{IκB}$ E3 ubiquitin ligase complex [23]. The ubiquitinated IκBα is degraded via the 26S proteasome [24], leading to exposure of the NLS of Rel A and nuclear translocation of the active p65:p50 complex (Fig. 9.2, left half). The expression of several IκB family members is regulated by the classical NF-κB pathway, which constitutes a negative feedback loop that prevents irreversible and sustained activation of classical NF-κB [25]. Newly synthesized IκBα enters the nucleus to bind to deacetylated p65:p50 heterodimers to shuttle them back to the cytoplasm [26, 27]. The IKK complex may also phosphorylate Rel A and p50 while still in the cytoplasm thereby enhancing their transcription activation potential [28].

Non-canonical NF-κB Pathway

Most of our knowledge about the processing of NF-κB2/p100 derives from genetic experiments with mice lacking or overexpressing a component of the alternative pathway. Processing of p100 is induced in B-cells by several stimuli such as CD40L [29], LTβ [30], BAFF [31], TNF-like weak inducer of apoptosis (TWEAK) [32], and RANKL [33], which is triggered by site-specific phosphorylation at two C-terminal serine residues (Ser-866 and Ser-870), mutation of which abrogates inducible processing. A kinase complex consisting of NF-κB-inducing Kinase (NIK) and IκB Kinase α (IKKα) phosphorylates p100, leading to polyubiquitination and proteasome-mediated processing (Fig. 9.2, right half). Phosphorylation of

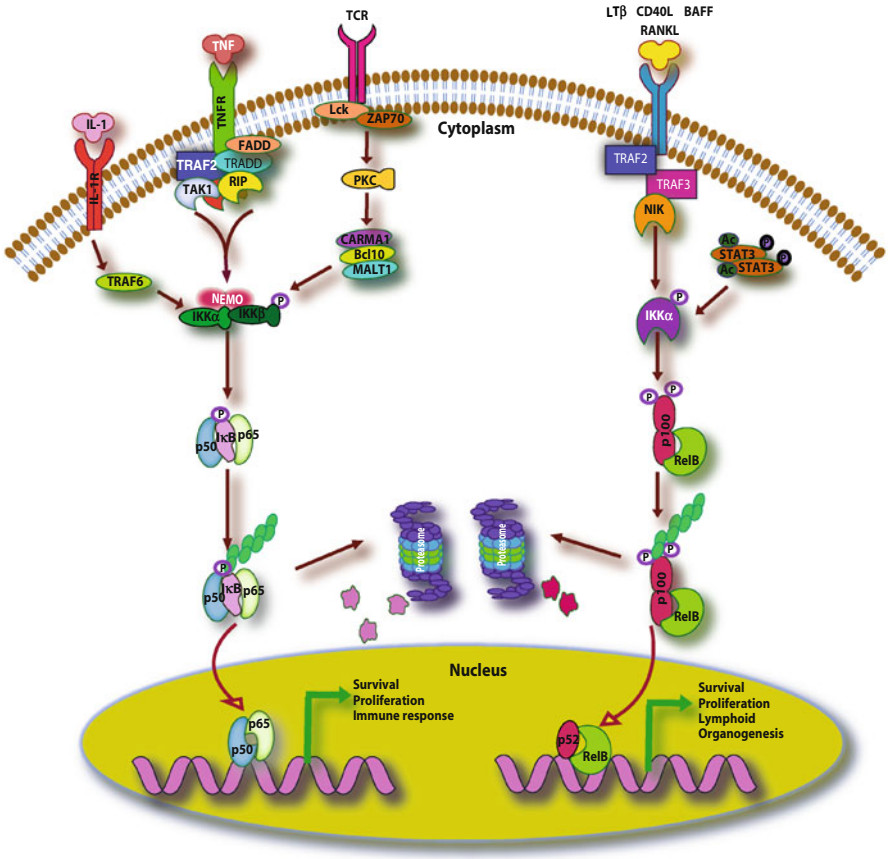

Fig. 9.2 Schematic representation of NF-κB signaling pathways. The *left* side of the figure represents the classical pathway, characterized by the nuclear translocation of the p65:p50 heterodimer upon activation by the heterotrimetric IKK complex. The *right* side of the figure represents the alternative pathway, characterized by the partial processing of p100, leading to nuclear translocation of the p52:Rel B heterodimer. The novel pathway downstream of activated STAT3 and activation of p100 processing is also depicted

serines 866 and 879 generates a binding site for the E3-ubiquitin ligase β-TrCP in the C terminus of p100, with the ubiquitin acceptor site being mapped to lysine residue K856 [34]. The $SCF^{\beta\text{-TrCP}}$ E3 ligase complex targets the polyubiquitinated p100 to the 26S proteasome, but due to the presence of a STOP signal consisting of a G-rich region (GRR), it undergoes partial degradation resulting in the release of the N-terminal p52 fragment bound to Rel B [35]. Unphosphorylated NF-κB2 precursor is retained in the cytoplasm in a complex with Rel B, and upon phosphorylation, ubiquitination and processing is translocated into the nucleus in the form of p52:Rel B dimers [36]. IKKα homodimers, nuclear translocation, and DNA binding are required for the constitutive processing of p100 [37]. Chromosomal rearrangements

that disrupt the *NFKB2* locus at 10q24 are associated with a variety of B- and T-cell lymphomas, including chronic lymphocytic leukemia (CLL), multiple myeloma, T-cell lymphoma, and cutaneous B- and T-cell lymphomas [38]. Although they differ molecularly, these rearrangements or deletions result in removal of the C-terminal inhibitory sequences of p100 and constitutive production of p52.

Sun and colleagues were the first to demonstrate involvement of a signaling protein in p100 processing by showing that overexpression of NIK in 293 cells was sufficient to induce p100 processing independently of IKKα or IKKβ [39]. In addition, splenocytes from *aly/aly* (Alymphoplasia) mice, which carry an inactivating point mutation in NIK, exhibit lower levels of p52 compared to their counterparts from *aly/+* heterozygous mice [40]. Moreover, overexpression of NIK triggered processing of p100 in wt MEFs as well as in IKKβ-deficient MEFS, but not in IKKα-deficient MEFs, indicating that IKKα is essential for p100 processing and that it acts downstream of NIK [41]. Moreover, stimulation of LTβR- or BAFFR/CD40-expressing cells with their cognate ligands induced p100 processing in wt cells but not in *aly/aly* cells [42]. These experiments provided conclusive evidence for a crucial role of NIK in inducible p100 processing [39]. NIK phosphorylates p100 at serines 866 and 870, which relieves the inhibition by processing inhibitory domain and also provides a docking site for IKKα [43, 44]. NIK also activates IKKα by phosphorylating it at serine 176 [45]. IKKα phosphorylates both the N-terminal (serines 99, 108, 115 and 123) and C-terminal (serine 872) phosphoacceptor sites, leading to recruitment of the $SCF^{\beta\text{-}TrCP}$ complex, Lysine 855 polyubiquitination and processing [44, 46]. In resting B cells, levels of NIK are very low as they are kept in check by complexing with TRAF proteins, which target NIK for ubiquitination and degradation. In cells stimulated by LTβ, CD40L and BAFF, degradation of TRAF2 and TRAF3 leads to stabilization of NIK, activation of IKKα, and processing of p100 [47].

The alternative NF-κB pathway plays significant roles in thymic organogenesis and self tolerance, secondary lymphoid organ development [48], B-cell development, survival, and homeostasis [38], and osteoclastogenesis [33, 49]. A variety of inflammatory disorders such as rheumatoid arthritis, Sjogren's syndrome, ulcerative colitis, multiple sclerosis, or chronic hepatitis follow disruption of the balance between cytokines and chemokines inducing or driven by the alternative pathway [50]. Blockade of this pathway may have therapeutic implications for diseases associated with chronic inflammation. Two mechanisms appear to play a role in NF-κB2-driven tumorigenesis: the overproduction of DNA-binding p52-containing complexes or the loss of inhibitory function of p100. In addition, Bcl-3, one of the main partners of p52, is overexpressed in a subset of human clinical breast tumor samples [51]. Bcl-3 may associate with p52:p52 homodimers and play an oncogenic role in breast cancer [52]. Moreover, the function of the tumor suppressor p53 may encompass regulation of the formation of Bcl-3:p52:p52 complexes. Wild-type p53 promotes the association of p52 homodimers with HDAC1 and also downregulates the expression of Bcl-3 [53]. Conversely, tumor-derived p53 mutants can induce expression of NF-κB2 [54], resulting in upregulation of anti-apoptotic genes and chemoresistance [53, 55]. Mutant p53 induces transcription of the NF-κB2 gene

and upregulates chemokine expression and cell migration via activation of the alternative pathway [56, 57]. Target gene expression by the p53 tumor suppressor can be regulated by p52, implying that p52 can regulate p53 function and influence p53-regulated decision-making following DNA damage and oncogene activation [58]. Thus, it is likely that abnormal or subverted activation of the alternative pathway may culminate in cellular transformation.

Germline knockout of *nfkb2* with no expression of either p100 or p52 displays severe defects in B-cell functions with impairment of the architecture of peripheral lymphoid organs [59], a phenotype shared by *aly/aly* mice [60], LTβR–κnockout mice [48], and IKKα-deficient mice [61]. Additional insights into the oncogenic role of NF-κB2 have resulted from the phenotype of *nfkb2*$^{\Delta CT/\Delta CT}$ mice that exhibit abnormal lymphocyte differentiation and develop spontaneous gastrointestinal tumors resulting in early post-natal death [62]. Importantly, p52 exhibits oncogenic potential by upregulating the expression of a subset of tumor-associated genes. Aberrant persistent processing of p100 is seen in T-cell leukemias associated with Human T-cell leukemia virus Type I (HTLV-1). The oncogenic event in this case is the virus-encoded oncoprotein Tax, which activates both the classical and alternative NF-κB pathways [63]. Other examples of virus-associated cancers that exhibit aberrant p100 processing are Kaposi's sarcoma (caused by KSHV-encoded oncoprotein vFLIP) [64, 65], Burkitt's lymphoma, and Hodgkin's disease (caused by EBV-encoded oncoprotein LMP-1) [66]. Some inducers of both classical and alternative NF-κB pathways generate two sequential waves of active NF-κB complexes [30] and thus, according to the cell type and the nature of the stimulus, the classical and alternative pathways control the fine tuning of their own gene as well as their target genes, which contribute to the pleiotropic biological functions of NF-κB.

NF-κB Proteins in PCa

Most studies of the subcellular localization of members of the NF-κB family in PCa have demonstrated an increase in nuclear localization of various subunits as progression occurs. In a study by Ross et al. (2004) where p65/Rel A was examined by immunohistochemistry, high expression of p65/Rel A was correlated significantly with higher mean preoperative serum PSA levels, mean tumor grade, nondiploid DNA content, and advanced stage and independently predicted biochemical disease recurrence [67]. In a study by Fradet et al. (2004), nuclear localization of p65/Rel A was found to be predictive of biochemical recurrence in patients with positive margins after radical prostatectomy [68]. In another study by Lessard et al. (2003), nuclear staining of p65/Rel A was found to be predictive of outcome independently from Gleason score. In addition, nuclear staining of p65/RelA was useful for progression risk stratification [69]. High levels of nuclear p65/Rel A were also found in lymph node metastases and in patients that developed bone metastases [70, 71]. High levels of nuclear p65/Rel A were also found to be correlated with biochemical relapse [72]. Activated p65/Rel A was shown o exhibit higher levels of expression

in PIN and PCa compared to benign prostatic tissues [11, 73]. Lessard et al. (2005) also performed a more systematic analysis of subcellular localization of all subunits of NF-κB in benign, PIN, and cancer tissues. This study showed the nuclear localization of Rel A, p50, Rel B, and p52 in 15.6, 10.5, 26.6, and 10.7 % of cancerous tissues respectively [74]. The authors predicted that the expression of p52:Rel B heterodimers is more frequent compared to the canonical Rel A:p50 heterodimers in prostate cancer tissues. More recently, high constitutive nuclear localization of Rel B was reported in human PCa specimens with high Gleason scores [75]. In addition, another study by Cai et al. (2011) showed that expression levels of NF-κB2 mRNA were significantly higher in cancerous tissues compared to BPH or benign tissues. Moreover, nuclear localization of p-p52 was observed in 42.6 % of PCa tissues, compared to 11.7 % of BPH and 6.7 % of benign tissues [76]. In contrast, nuclear localization of p-p50 tended to decrease from benign to BPH to PCa tissues, implying that activation of the alternative pathway of NF–κB may be more frequent in PCa than previously reported, and that nuclear localization of p52 in PCa may be a predictor of poor outcome. A study by Seo et al. (2009) showed that nuclear localization of p50, p52, and Rel A was observed in 28, 18.7, and 37.4 % of PCa specimens respectively, while no nuclear staining was detected in benign or PIN tissues [77].

The above studies showcase the variable results and divergent conclusions from immunohistochemical analyses, which may be due to the selection of tissues, antibodies, methods of tissue preservation, etc. Hence, the importance of a large study with careful selection of antibodies and tissues to assess the relative significance of the classical and alternative pathways in different stages of PCa cannot be overemphasized. The common theme that emerges from the above reports is that nuclear localization of classical and alternative NF-κB subunits is detected at higher rates in PCa compared to benign prostates, lending credence to the oncogenic role of both pathways of NF-κB activation in PCa.

Classical NF-κB in PCa

Several commonly used PCa cell lines exhibit higher nuclear levels of classical NF-κB subunits and higher levels of DNA-binding by p65:p50 heterodimers. Intriguingly, most of the PCa cell lines that exhibit chronic NF-κB activation in vitro either lack AR or have low levels of AR. This observation led to experiments which showed that the AR and p65/Rel A regulate each other by mutual transcriptional interference [78, 79]. Transiently coexpressed AR and p65/Rel A were able to transrepress each other's transcriptional activity in COS-1 cells [78]. It has been postulated that AR either activates or represses classical NF-κB, depending on the availability of androgens, where AR activates NF-κB in the absence of androgens and represses it in the presence of androgens [80]. On the other hand, AR is a transcriptional target of classical NF-κB and overexpression of p65/Rel A increases expression of AR [81]. The AR promoter contains three κB sites which have

differential effects on AR expression depending on occupancy by p65:p50 heterodimers. In rat hepatocytes where the κB2 site in AR promoter is occupied by transcriptionally inactive p50:p50 homodimers, expression of AR is suppressed [79], while in Sertoli cells where the κB sites are occupied by the p65:p50 heterodimers, AR expression is induced [82]. Another report showed that p65/Rel A binds to a repressive κB element in the AR-responsive core enhancer promoter and inhibits AR-mediated PSA transactivation [83]. These ambiguous results showing either positive or negative effects of AR and classical NF-κB on each other may be due to the variety of experimental approaches employed: transient transfections, non-prostatic cell lines, etc. Affirmation or negation of these results can only be achieved by genetically engineered mouse models, which will yield definitive answers to the question of the nature of interaction between classical NF-κB and AR.

Interestingly, even though the nature of interaction between classical NF-κB and AR is still in question, the effect of activation of classical NF-κB is unequivocal. Constitutive activation of classical NF-κB is observed in many cell lines, mouse models, and human tissues of PCa [67, 77]. The classical NF-κB pathway has been shown to control PCa progression to a castration-resistant state in a mouse model [84], and nuclear localization of p65/Rel A was shown to predict biochemical recurrence in patients with positive margin PCa [68]. Classical NF-κB also induces expression of PSA by binding to a κB element in its promoter and is upregulated in CRPC [85]. Thus, results of these investigations into the functionality of classical NF-κB in PCa have been dependent on experimental approaches where a clear picture is yet to emerge.

Non-canonical Pathway in PCa

Even though the processing of p100 in lymphoid cells has been well characterized, the stimuli and mechanisms involved in higher expression of the p52 subunit in non-lymphoid cells and solid tumors have only recently begun to be elucidated. A report by Nadiminty et al. (2006) unveiled a novel pathway of p100 processing mediated by activated STAT3, implicating activated, but not latent, STAT3 in the induction of p100 processing in PCa cells (Fig. 9.2). Both latent and activated STAT3 can complex with p100, but only STAT3 activated by phosphorylation and acetylation can induce processing by recruiting IKKα to the complex [86]. This pivotal role of STAT3 in p100 processing may also be true in other solid tumors where the factors responsible for phosphorylation (JAK2) and acetylation (CBP/p300) of STAT3 are ubiquitously available. Other reports also show the important role of activated STAT3 in maintenance of NF-κB activity in tumor cells [87].

Previous studies have focused on the classical pathway due to the sheer volume of information available, while emerging new evidence points to a key role of the alternative pathway in PCa. Lessard et al. (2007) showed that p100 processing and p52 nuclear accumulation are stimulated by the AR in PCa cells [88]. A study by Nadiminty et al. (2008) demonstrated that NF-κB2/p52 is overexpressed in PCa

and induces castration-resistant growth of androgen-sensitive LNCaP PCa cells both in vitro and in vivo [89]. Another report also delineated the mechanisms involved in the ability of NF-κB2/p52 to induce castration-resistant growth, which is derived from the aberrant activation of AR under androgen-depleted conditions [90]. In this study, the overexpression of NF-κB2/p52 in androgen-sensitive LNCaP cells enhanced expression of classical AR target genes, PSA and NKX3.1 by recruiting higher levels of AR to their gene promoters. In addition, the report also showed that NF-κB2/p52 activates the AR and enhances nuclear translocation and activation of AR by interacting with its NTD. NF-κB2/p52 enhances the recruitment of coactivators such as p300 to the promoters of AR-dependent genes, resulting in increased transactivation of AR-responsive genes under androgen-deprived conditions. The upregulation of PSA expression by NF-κB2/p52 requires the expression of the AR, and suppression of p52 expression leads to inhibition of AR activation under conditions resembling castration. Downregulation of NF-κB2/p100 using shRNA resulted in reduced production of p52 and decreased AR transactivation ability and castration-resistant growth in C4-2 CaP cells [90]. Notably, unlike DHT-activated AR, p52-activated AR binds, not to the proximal and distal enhancer elements in the PSA promoter, but only to the distal enhancer region, indicating that p52-activated AR may be in a different conformational state compared to the DHT-activated AR. These findings, together with previous reports that levels of p52 are elevated in prostate cancer cells and that NF-κB2/p52 promotes prostate cancer cell growth in vitro and in vivo, suggest that p52 may play a critical role in the progression of CRPC.

Genome-wide microarray analysis revealed the regulation of several anti-apoptotic and pro-survival genes by NF-κB2/p52 in PCa cells [91]. The expression levels of pro-angiogenic and pro-metastatic genes such as PLAU, Twist2, VEGFC, and CXCL1 were upregulated by NF-κB2/p52 in LNCaP PCa cells, indicating that p52 activation may have a broader range of effects on CRPC progression. More recent reports from the same group have also shown that AR expression is suppressed by miRNA-let-7c, which is mediated by its master negative regulator, Lin28 [92, 93]. NF-κB2/p52 upregulates expression of Lin28 in PCa cells by transcriptional activation, and miRNA-let-7c is downregulated in PCa due to activation of Lin28 by NF-κB2/p52. Furthermore, NF-kB2/p52 induces expression of genes encoding steroidogenic enzymes such as AKR1C3, HSD3B2, CYP17A1, SRD5A1, etc. Upregulation of steroidogenic enzyme expression by p52 results in elevated levels of intracellular androgens in PCa cells in vitro and in vivo (Nadiminty et al., Proceedings of Annual Meeting of the AUA 2011). These findings may help clarify the multifaceted role of NF-κB2/p52 in interaction with AR in PCa and support the feasibility of targeting both the canonical and alternative pathways of NF-κB as a means of controlling CRPC progression.

Several studies report the crucial roles of different stimuli/components of the alternative pathway in development of CRPC. For instance, an epidemiological study revealed that reduced PCa risk due to consumption of nonsteroidal anti-inflammatory drugs, such as aspirin, is limited to men who express a common

polymorphic LTα allele that specifies high lymphotoxin production [94]. Furthermore, B-cell-derived lymphotoxin β signaling activates IKKα and STAT3 to promote survival of androgen-deprived PCa cells, suggesting that patients who produce high levels of lymphotoxin are more susceptible to castration-resistant progression of PCa [95]. IKKα, a kinase essential in the alternative pathway, was demonstrated to control the activity of mammalian target of Rapamycin (mTOR) in Akt-active, PTEN-inactive PCa cells [96], which implicates at least a partial activation of the alternative pathway. Further, a mutation that prevents IKKα activation was demonstrated to slow PCa growth and inhibit metastasis in TRAMP mice. Decreased metastasis correlated with elevated expression of the metastasis suppressor Maspin, the ablation of which restored metastatic activity. IKKα activation by RANKL inhibited Maspin expression in prostate epithelial cells, whereas repression of Maspin transcription requires nuclear translocation of active IKKα. The amount of active nuclear IKKα in mouse and human prostate cancer was shown to correlate with metastatic progression, reduced Maspin expression, and infiltration of prostate tumors with RANKL-expressing inflammatory cells, implying that tumor-infiltrating RANKL-expressing cells lead to nuclear IKKα activation and inhibition of Maspin transcription, thereby promoting the metastatic phenotype [97]. IKK1 was also implicated in the regulation of AR nuclear translocation and activity as well as expression [98, 99]. A few reports also show that CRPC growth and maspin expression in PCa are controlled by Rel B [75, 100], which in turn is regulated by NF-κB2 [101]. Even though these studies describe the activities of these intermediates as being independent of activation of p52-containing complexes, it is difficult to visualize a scenario of nonactivation of the alternative pathway upon stimulation by lymphotoxin or upon activation of IKKα. Hence, whether the findings reported in the above studies implicate activation of p100 processing and p52 production is debatable and remains to be explored further. Conditional and tissue-specific knock-in of intermediates of the alternative NF-κB pathway would facilitate the understanding of their role in specific tissues or cell types. Such models will prove to be crucial for examining the roles of p52-containing complexes in different diseases and for determining which target genes are regulated by particular p52-containing dimers.

Targeting

Given the importance of the NF-kB pathway in a multitude of biological processes, an impressive body of evidence implicates NF-κB activation in the development of lymphoid-, myeloid-, and epithelial-derived malignancies. It is also important to note that both the classical and alternative pathways have many nodes at which targeting strategies may be devised: for instance, the classical pathway may be targeted at the levels of activation of IKKs, degradation of IκBα, nuclear translocation of p65:p50 dimers, etc. The alternative pathway may be targeted at levels of

TRAF3 degradation, NIK activation, IKKα activation, p100 processing, nuclear translocation of p52-containing dimers, etc. Numerous inhibitors of NF-κB are under development or have been developed. Small molecule inhibitors of IKK, proteasome inhibitors, IκBα super-repressor, and other viral-encoded inhibitors of other components of the NF-κB activation pathway have been shown to induce apoptosis and inhibit the proliferation of tumors or tumor-derived cell lines [102]. Unfortunately, none of the small molecules have proven to be completely specific for IKK or NF-κB, and viral vectors are not yet practical for clinical applications. Anticarcinogenic and chemopreventive effects of phytochemicals such as resveratrol, curcumin, silibinin, and plant phenolics have been explored [103, 104] and though impressive, the results are likely to be difficult to translate into preclinical and clinical stages, due either to toxicity issues or formulation issues. Clearly, more specific and potent inhibitors are needed for the dream of targeting NF-κB in cancer to be realized.

Due to the more cell-specific and context-specific role of the alternative pathway, targeting its components may have therapeutic advantages in certain diseases, but much remains to be learned about the synergism between the alternative NF-κB pathway and the classical NF-κB pathway and other transcription factor-driven signaling events [10]. Moreover, care should be exercised not to interfere with the pro-apoptotic and anti-oncogenic activity of p100 [105] while inhibiting the anti-apoptotic activity of p52-containing dimers. Inhibition of NF-κB2/p52 by shRNA inhibits PCa cell growth [90]. Inhibition of IKKα activity by a pharmacological inhibitor inhibits AR activity and increases apoptosis in PCa cells [98]. Phytochemicals that target inflammatory reactions may conceivably be useful for inhibiting components of the alternative pathway [106]. Thus, a closer look at targeting the alternative pathway is warranted given the emerging evidence on the importance of p52-containing dimers in different cancers and the importance of IKKα activation in epithelial differentiation and osteoclastogenesis.

Conclusions and Future Perspectives

Since the discovery of the alternative NF-κB pathway more than a decade ago, enormous progress has been made in understanding its biological regulation and functions, which has made rational drug design in the treatment of inflammation-related disorders and in cancer possible [107]. But questions remain regarding the stimuli activating p100 processing and the downstream intermediates in non-lymphoid malignancies such as PCa. Some of the questions that still need to be answered are the following: *Is NIK overproduced or hyperactivated in CRPC?; Is the level of TRAF3 low in CRPC?; Is the activation of IKKα independent of NIK in CRPC?; What are the biological reasons for elevated p100 processing in CRPC?* More information is needed about the cell type- and stimulus-specific activation and promoter-binding of p52-containing dimers in PCa and in other cancers to realistically envisage the use of preclinical therapeutics in clinical trials.

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Chapter 10
The Functional Role of DAB2IP, a Homeostatic Factor, in Prostate Cancer

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Abstract Maintenance of cellular architecture and normal physiologic functioning of the prostatic epithelium is dependent on androgen. Cell proliferation and cell differentiation in normal prostatic gland maintains a homeostasis. This homeostatic control is impaired in prostate cancer (PCa) cells. Therefore, dissecting the mechanism of homeostatic machinery will provide a better understanding of PCa and allow us to formulate effective strategies for cancer therapy.

One of the most exciting developments in oncology has been the step-by-step construction of signaling cascade that traces the path of extracellular stimuli, all the way from the external membrane to the cell nucleus. DAB2IP was first identified as a unique RAS-GTPase activating protein from the basal cell population in prostate. Loss of DAB2IP is frequently detected in PCa. DAB2IP modulates different signal cascades associated with cell proliferation, survival, and apoptosis. Restoring DAB2IP expression in PCa can inhibit cancer metastasis by preventing epithelial-to-mesenchymal transition that is considered to be a cell de-differentiation process. In addition, DAB2IP can inhibit angiogenesis by enhancing endothelial apoptosis and/or inhibiting vascular growth factor and its receptor expression. Consistent with

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these findings, DAB2IP can also inhibit the onset of the stem cell phenotype of PCa cells. Taken together, DAB2IP appears to be a key factor in controlling homeostasis of prostatic epithelium.

Homeostasis of Normal Prostate Epithelium and Prostate Oncogenesis

The prostate gland is a typical androgen-dependent (AD) organ. Large transient surges of serum androgen, estrogen, and progesterone levels normally occur very early in postnatal life. Castration of neonatal mice or rats greatly inhibits continued growth and development of the prostate, an effect that can be reversed by administration of androgen. However, administration of exogenous androgen to immature males accelerates prostatic growth so that maximal prostatic size is achieved precociously [1, 2]. However, once maximal size is attained, additional androgen treatment does not elicit a further increase in the number of prostatic epithelial cells [1, 2], indicating the presence of homeostatic machinery in prostatic epithelium during prostate development.

Tissue degeneration is a basic biologic phenomenon that occurs in a variety of tissues under certain physiologic and pathologic conditions. The prostate gland requires androgen for maintenance of its normal physiologic function and has been used as a model system for studying the process of organ degeneration [1, 2]. In prostatic epithelia, there are two major histomorphologically distinct cell types, i.e., luminal and basal. The luminal epithelial cells are often referred to as highly differentiated because they have specialized secretory functions. After castration these cells undergo apoptosis, and the gland involutes. In contrast, the basal cells are thought to be less well differentiated and androgen-independent (AI), because basal cells still survive in the involuted gland after castration [3–5]. In the involuted gland, androgen is the key factor for regenerating the normal acini/ductal structure and the function of the prostate gland by promoting differentiation of the remaining basal cell population [6–8]. Despite repeat administration of androgen to castrated animals, the prostate always regrows to a previously programmed organ size, suggesting that a homeostatic machinery is operational in the basal cell population. Interestingly, the basal cell population is frequently lost in prostate cancer (PCa) [9, 10]. Nevertheless, the nature of such a homeostatic machinery remains undefined.

Primary PCa is an androgen-dependent (AD) disease; however, PCa eventually acquires AI status in patients who have received androgen deprivation therapy. This stage of the disease is known as castration-resistant PCa (CRPC) because the tumors become resistant to conventional therapeutic regimens. CRPC tumors often express many markers that are associated with the basal cell population [11–13] but with malignant phenotypes. Therefore, it is very likely that the altered homeostatic machinery is associated with CRPC.

Identification of DAB2IP from Normal Prostate Basal Cell Population

To search for this potential homeostatic machinery, we employed a differential display polymerase chain technique using basal cell-enriched tissue from degenerated prostate and tissue from intact prostate. We discovered that the DOC-2/DAB2 protein complex from basal cells is involved in the regulation of the growth and differentiation of prostatic epithelium [14]. Subsequently, DAB2IP (DIP1/2 or AIP) was identified as a DOC-2/DAB2-interactive protein by yeast two-hybrid screening. Cloning of a full-length cDNA revealed that DAB2IP contains an open-reading frame of 996 amino acids with a calculated molecular mass of 110 kDa and a pI of 6.76. Analysis of the amino acid sequence of human DAB2IP suggested that it contains several functional domains, including an N-terminal pleckstrin homology (PH) domain (aa 20–70) with a high affinity for phosphoinositides, a C2 domain (aa 90–120) involved in binding phospholipids in a calcium-dependent or -independent manner, a RAS-GTPase-activating domain (RAS-GAP) (aa 198–397), a C-terminal period-like (PER) domain (aa 522–719) involved in binding to the intact RING finger of TNF-receptor-associated factor 2 [15], a proline-rich (PR) domain (aa 796–805) involved in interacting with proteins containing an SH3 domain, and a leucine zipper (LZ) domain (aa 842–861), which is a protein–protein dimerization domain. DAB2IP appears to be a novel member of the RAS–GAP family of proteins.

RAS proteins are involved in many cell functions including growth, differentiation, survival, and cytoskeleton organization. Mutation of any one of many identified amino acids can produce a highly oncogenic protein. Normal p21ras (RAS) exists in equilibrium between an active form (binds to GTP to form GDP.RAS) and an inactive form (hydrolyzes GTP to form GDP.RAS). The rate of GDP release and GTP hydrolysis is regulated by two distinct groups of proteins: guanine nucleotide exchange factors (i.e., GEF) that catalyze the release of bound GDP, and GAPs that increase the rate of GTP hydrolysis. In PCa, RAS activation has been reported to be particularly prevalent in high-grade tumors. However, *Ras* gene mutation or alteration is rarely associated with PCa [16, 17], suggesting that some other effector proteins such as GAP may be defective.

In normal prostate, DAB2IP expression is elevated and associated with the basal cell population following androgen deprivation. Biochemically, DAB2IP exhibits a typical GAP activity similar to other Ras GAP proteins (i.e., $p120^{GAP}$) by stimulating GTPase activity of H-Ras, K-Ras, R-Ras, and TC21 but not Rap1A. DAB2IP is able to inhibit mitogen-induced signal transduction and growth of PCa cells. The expression of DAB2IP is either downregulated or lost in several PCa cell lines as well as in PCa clinical specimens.

Altered Regulation of *DAB2IP* Gene Expression in PCa

Data from both tissue culture models and clinical specimens of PCa demonstrate that the downregulation of DAB2IP expression is associated with the progression of PCa [18, 19]. The human *DAB2IP* gene is located at 9q33.1–q33.3 and spans ~96 kb with 15 exons and 14 introns. The *DAB2IP* promoter contains no canonical TATA box [20]. Although *DAB2IP* gene mutation or deletion has not been found in PCa, *DAB2IP* mRNA in PCa is significantly lower than its normal counterpart. Indeed, DNA hypermethylation of the core promoter sequence is detected in PCa cells [20, 21] and a similar pattern is found in other cancer types as well [22–24]. We have found that the histone deacetylase inhibitor (trichostatin A) and the DNA hypomethylation agent (5′-aza-2′-deoxycytidine) act cooperatively in increasing DAB2IP gene expression in PCa cells [21], indicating that DNA methylation and histone acetylation are key regulatory mechanisms for *DAB2IP* gene expression in PCa. Moreover, histone methylation plays an additional role in modulating *DAB2IP* gene expression. For example, the *DAB2IP* gene promoter is repressed by a human homolog of the Drosophila enhancer of the zeste gene (EZH2) [25] that encodes a histone lysine methyltransferase and is often elevated in metastatic PCa [26]. Thus, altered *DAB2IP* gene expression in PCa is primarily controlled by epigenetic regulation. Moreover, a study [27] of an acute myeloid leukemia patient with a t(9;11)(q34;q23); found that the intron 9 of the MLL gene was translocated into the exon 2 of DAB2IP, which resulted in disruption of the pleckstrin homology (PH) domain in the DAB2IP protein, suggesting that gene fusion of DAP2IP may be another mechanism for disrupting DAB2IP function. So far, no similar finding has been reported in PCa patients.

In contrast, we found that decreased DAB2IP protein expression can be regulated by an oncogene Skp2 (S-phase-associated kinase protein-2)-mediated proteasome degradation. Skp2 is a member of the Skp, Cullin, F-box-containing complex (or SCF complex) [28], which is an ubiquitin E3 ligase that has been reported to be elevated or overexpressed in PCa specimens [29, 30]. Increased copy numbers of the Skp2 gene have been reported in advanced or metastatic PCa [31]. We found that there is an inverse correlation between DAB2IP and Skp2 protein expression in several immortalized human normal prostate cell lines such as PNT1A and PZ-HPV-7. DAB2IP protein is elevated in a time-dependant manner following treatment of a PCa cell line (PC-3) with the proteasome inhibitor MG132. Such Skp2-mediated DAB2IP protein turnover is associated with proliferation of androgen-independent PCa cells (C4-2). We further demonstrated that the N-terminal end of DAB2IP, particularly the C2 and GAP domains, interacts with Skp2 as a major ubiquitination site. Interestingly, DAB2IP is able to modulate Skp2 protein degradation through the Akt pathway, where DAB2IP can inactivate Akt by suppressing PI3K activity [32] and active Akt is known to prevent Skp2 from APC/Cdh1-mediation degradation [33]. Immunohistochemical staining (IHC) for both DAB2IP and Skp2 using a human PCa tissue array showed an inverse relationship between DAB2IP and Skp2 protein expression in ~40 % of PCa specimens (26.2 %

of DAB2IP^{high}-Skp2^{low} and 14.1 % of DAB2IP^{low}-Skp2^{high}). Therefore, this reciprocal relationship of Skp2 and DAB2IP represents a delicate balance between oncogene and tumor suppressor in the normal cell, which could result in an oncogenic process once the balance is altered.

The Role of DAB2IP in Prostate Oncogenesis

Accumulating evidence has demonstrated that the *DAB2IP* gene plays a crucial role in maintaining cell homeostasis, while its loss will lead to oncogenesis of normal prostate epithelial cells and promote more aggressive phenotypes (i.e., cell growth, cell survival and apoptosis, and invasion and metastasis) in PCa [18–20, 32, 34–36]. Furthermore, the homeostatic machinery of DAB2IP in prostate cells has recently been dissected (Fig. 10.1), in which multiple functional motifs are involved in the regulation of a wide spectrum of biological functions (Fig. 10.2).

Endogenous DAB2IP is highly expressed in immortalized human normal epithelial cells, such as PZ-HPV-7, RWPE-1, and PNT1A [20]. In general, these cells do not form anchorage-independent colonies or tumors in a xenograft animal model.

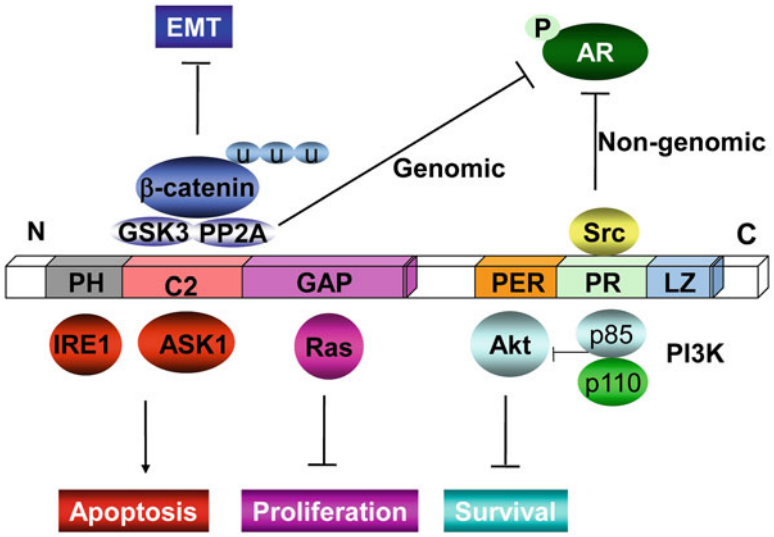

Fig. 10.1 *The biologic functions of DAB2IP protein domains*. The PH domain can recruit IRES and the C2 domain interacts with ASK1, which is involved in enhancing apoptosis. The C2 domain can interact with PP2A to activate GSK3β, which can block Wnt-mediated EMT process. Also, PP2A can dephosphoryate AR, which suppresses AR transcription activities. The GAP is able to inhibit RAS activities involved in cell proliferation. The PR domain is able to interact Src and inhibit its activity, which leads to the inhibition of nongenomic activation of AR. In addition, the PR can recruit regulatory domain (p85) of PI3K, then suppress the activity of PI3K catalytic domain (p110), and the PER domain can recruit Akt leading to the inhibition of cell survival

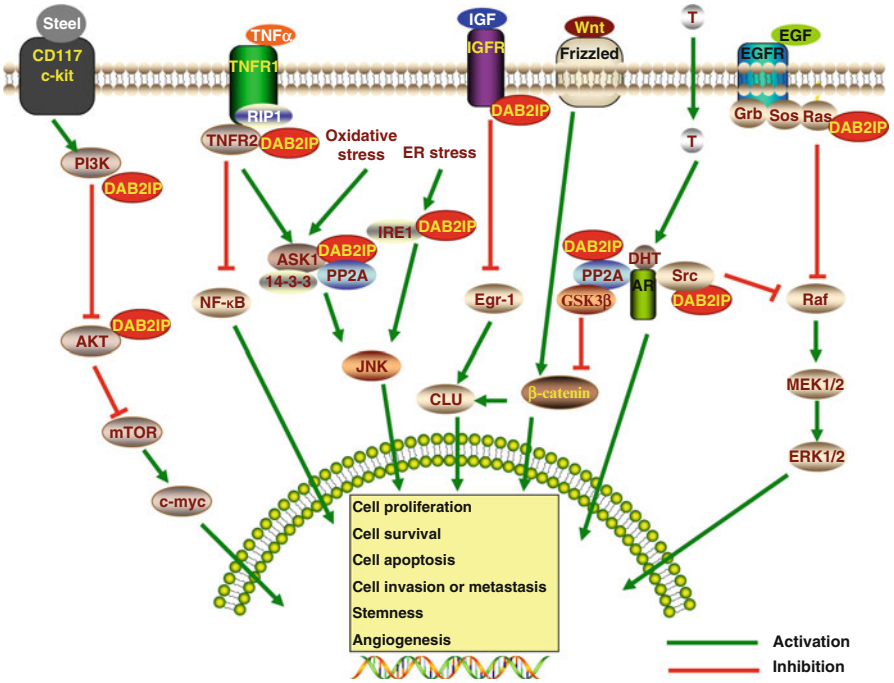

Fig. 10.2 *The interaction of DAB2IP with different signal pathways*. DAB2IP protein functions as signalosome platform for crosstalk between various signal pathways. In general, DAB2IP suppresses the signal signaling leading to cell proliferation, survival, cancer invasion and metastasis, cancer stemness, and angiogenesis. However, DAB2IP is able to enhance apoptosis signaling induced by TNF-α, oxidative stress, or ER stress. Overall, by modulating these pathways, DAB2IP is able to maintain the homeostasis of normal cell

However, PZ-HPV-7 cells formed anchorage-independent colonies and subcutaneous xenografts in severe combined immunodeficiency (SCID) mouse when the endogenous DAB2IP is stably knocked down by a small hairpin RNA (shRNA) (unpublished data). Similarly, Min et al. [19] have also reported that DAB2IP silencing will drive malignant transformation in primary human prostate epithelial cells PrECs and mouse embryonic fibroblasts (MEFs). For example, PrECs cells immortalized by SV40 T antigen and androgen receptor (AR) are unable to form anchorage-independent colonies. However, DAB2IP ablation promotes colony growth of PrECs cells infected with lentiviruses expressing DAB2IP-specific shRNA. In vivo, mice injected with PrECs expressing DAB2IP shRNA develop PCa that express prostate-specific antigen (PSA). Noticeably, DAB2IP-deficient tumors and oncogenic H-Ras^{V12}-expressing tumors show similar growth kinetics; all are highly proliferative with an increased expression of Ki-67 with very little sign of apoptosis [19].

DAB2IP was first identified as a novel GAP protein containing a Ras–GAP homology domain [20] that spans residues 177–409, which is commonly seen in all

members of the Ras–GAP family. Using a tumorigenic human PCa cell line (i.e., C4-2) with no detectable endogenous DAB2IP, we observed that ectopic expression of DAB2IP significantly inhibits in vitro cell growth [35]. To further demonstrate the unique function of the GAP domain, a DAB2IP mutant construct with a single amino acid mutation (R220L), which disrupts GAP activity, exhibits loss of growth inhibitory effect on PCa cells [19, 35]. These results indicate that DAB2IP is a typical Ras GAP that is critical for the inhibition of cell proliferation of PCa cells stimulated by many peptide growth factors.

Androgen and its receptor-meditated signal pathways have been studied extensively in PCa and are considered to be the most crucial driving forces for prostate oncogenesis [37]. We found that DAB2IP can modulate androgen-induced cell proliferation in prostate cells. For example, dihydrotestosterone (DHT) could stimulate the growth of C4-2 cells in a dose-dependent manner; however, DHT failed to stimulate the proliferation of DAB2IP-expressing C4-2 cells. In contract, cell proliferation significantly increased in DAB2IP knockdown of PZ-HPV7 cells treated with DHT in a dose-dependent manner [38]. Similarly, in DAB2IP knockout (i.e., $DAB2IP^{-/-}$) mice, the hyperplastic gland is associated with elevated nuclear AR staining and activated AR-mediated signaling. Furthermore, based on IHC of tissue arrays, DAB2IP expression inversely correlates with the AR activation status, particularly in recurrent or metastatic PCa specimens.

We dissected the mechanism of action of DAB2IP with respect to AR function. First, DAB2IP can inhibit the nongenomic pathway of AR. Androgen binding can result in AR-mediated activation of the Ras/extracellular signal-related kinase (ERK) pathway through nongenomic activation of the c-Src tyrosine kinase [39–42]. For example, DHT stimulated a rapid activation of c-Src and Erk evidenced by the elevated phosphorylation of Src Tyr416 ($Y416^{P}$) and Erk Thr202/Tyr204 ($Thr202/Tyr204^{P}$) after 5 ~ 15 min of treatment. However, the presence of DAB2IP in C4-2 cells showed a dramatic inhibition of c-Src ($Y416^{P}$) as well as an inhibition of Erk ($Thr202/Tyr204^{P}$). Moreover, knockdown of DAB2IP in PZ-HPV7 cells could significantly increase both phosphorylation levels of c-Src ($Y416^{P}$) and Erk ($Thr202/Tyr204^{P}$) after DHT stimulation. In contrast, increased expression of constitutively active v-Src in DAB2IP-expressing C4-2 cells was able to antagonize its growth. Consistent with our observation, Min et al. [19] have also reported that knockdown DAB2IP in PrECs cells could significantly elevate the RasGTP expression and Erk Thr202/Tyr204 phosphorylation, while re-expression of DAB2IP will reverse this activation.

Mechanistically, AR is known to interact with Src via its proline-rich (PR) domain [39–42]; this interaction further activates Erk phosphorylation and its downstream signaling. The PR domain has been well characterized as an SH3-interacting domain critical for signal transduction elicited by peptide growth factor receptors [43, 44]. In general, protein–protein interaction via the PR domain can relay a signaling cascade to downstream effectors. Noticeably, the PR domain in DAB2IP contains a ten-proline repeat that is distinct from the other two classes of well-characterized PR consensus sequences (RxxPxΦP or PxΦPxR; x is any amino acid and Φ is hydrophobic amino acid). Particularly, we found that the PR domain of

DAB2IP can interact directly with c-Src by competing with AR binding to c-Src; such competition inhibited c-Src activation leading to downstream Erk phosphorylation. Unlike other known PR domains, this unique PR domain in DAB2IP plays an inhibitory role in signaling transduction.

DAB2IP can also inhibit the genomic pathway of AR by preventing nuclear translocation, phosphorylation, and transcription activity of AR. We observed that DAB2IP is capable of decreasing androgen-mediated AR nuclear translocation by inhibiting serine 81 phosphorylation of AR and its gene induction, in which DAB2IP recruits protein phosphatase 2 (PP2A) to dephosphorylate AR (Fig. 10.1). In addition to classic androgen ligand, DAB2IP is able to suppress AR activities in an androgen-independent manner. For example, DAB2IP can inhibit Wnt-elicited AR activation as well as several AR splice variants in PCa cells. Thus, we believe that loss of DAB2IP underlies the AR hyperactivation often associated with the onset of castration-resistant prostate cancer (CRPC).

In addition to its antiproliferation activity, DAB2IP is also involved in cell apoptosis. In endothelial cells undergoing apoptosis after TNF-α treatment, the presence of DAB2IP facilitates this process by dissociating apoptosis signal-regulating kinase 1 (ASK1) from its inhibitor 14-3-3 via the pleckstrin homology (PH) and C2 domains of DAB2IP [15, 45]. In addition to its activity to enhance TNF-α-induced ASK1-JNK signaling, DAB2IP can regulate the endoplasmic reticulum (ER) stress-induced, but not oxidative stress-induced, ASK1-JNK activation that is related with cell apoptosis in MEFs and vascular endothelial cells [46]. Mechanistically, ER stress induced formation of DAB2IP–inositol-requiring enzyme-1 (IRE1) complex, and the PH domain of DAB2IP is critical for the IRE1 interaction, which will facilitate IRE1 dimerization and lead to the activation of JNK/XBP-1 axis [46] (Fig. 10.2). A similar biological function of DAB2IP can be found in PCa cells. Our recent publication has shown that DAB2IP is a pro-apoptotic regulator in PCa cells treated with TNF-α or PI3K inhibitor LY294002 [32] in which DAB2IP has a dual function by activating ASK1 activities to enhance cell apoptosis and by inhibiting PI3K-Akt activities to suppress cell survival. Similarly, both activated ASK1 or inactivated PI3K-Akt was observed in glandular epithelia from DAB2IP$^{-/-}$ animals [32] and in primary prostate epithelial cells [19]. Different from its effects in activating ASK1, DAB2IP inhibits both PI3K and Akt activities by directly interacting with these proteins via the PR or PERIOD-like (PER) domain [32]. For example, DAB2IP phosphorylation at S604 is critical for changing DAB2IP conformation, and the PR domain of DAB2IP is able to sequester the regulatory subunit (p85) of PI3K and suppress the activity of catalytic subunit (p110) of PI3K (Fig. 10.1). Apparently, the PR activity in DAB2IP indicates that it is a new class of PR domain in terms of its inhibitory signal transduction via PR-SH3 domain interaction. In addition, the PER domain of DAB2IP can interact with Akt resulting in a reduction of Akt phosphorylation [32]. DAB2IP appears to be a unique scaffold protein capable of cross talk with a variety of effector molecules involved in different signaling cascades ranging from cell growth, survival, and apoptosis, which are critical pathways found to be associated with prostate oncogenesis (Fig. 10.2).

The Mechanism of DAB2IP in the Invasion and Metastasis of PCa

Using genome-wide association data, a single nucleotide polymorphism probe (SNP rs1571801) in the first intron region of *DAB2IP* gene has been discovered to correlate with a high risk of aggressive PCa [20, 21]. Moreover, significantly lower DAB2IP expression is detected in high-grade PCa tissues [34]. Thus, DAB2IP is likely involved in PCa progression. Indeed, loss of DAB2IP expression in PCa cells correlates with an onset of epithelial-to-mesenchymal transition (EMT) based on morphologic change, switching expression of an epithelial marker (i.e., E-cadherin) to a stromal marker (i.e., vimentin), increased cell invasion and lymph node invasion in orthotopic xenograft models. Conversely, restoring DAB2IP in metastatic PCa cells reverse EMT to become mesenchymal-to-epithelial transition (MET), indicating that EMT or MET is a transient process. In DAB2IP$^{-/-}$ mice, prostate epithelial cells exhibited elevated mesenchymal markers, which is characteristic of EMT in vivo. In addition, there is an inverse correlation between DAB2IP and EMT marker protein expression in PCa specimens. Since Wnt is known as a critical EMT regulator, we have analyzed the role of DAB2IP in this process and found that DAB2IP could recruit PP2A to active GSK-3β through its C2 domain and prevent β-catenin from entering nuclei to modulate genes required for the EMT phenotype [36] (Fig. 10.1).

Consistent with our observation, Min et al. used a different orthotopic xenograft model to demonstrate that loss of DAB2IP could promote metastatic PCa [19]. Interestingly, the H-Ras^{V12}-driven PrECs tumors remained noninvasive and never disseminated, but DAB2IP-deficient tumors were invasive and metastatic. These tumors invaded the hip, lumbar muscle, and bladder and developed widespread metastasis in liver, proximal and distal lymph nodes, seminal vesicles, testes, and vas deferens, while no metastases were observed from cells with restored *DAB2IP* gene expression. Notably, DAB2IP knockdown in PrECs is able to promote EMT through different signaling pathways, in which DAB2IP deletion coordinately activates both Ras and NF-κB. Min et al. demonstrated that a DAB2IP double mutant (R289L and S604A) became more potent in promoting invasion, EMT, and activating the NF-κB pathway, indicating that these two pathways work cooperatively in these events through loss of specific functions of these two DAB2IP domains. Of great significance, this study also integrated the polycomb-group gene (i.e., EZH2)-mediated epigenetic silencing of DAB2IP and its protein function in modulating cancer metastasis, which further supports the importance of these signaling cascades in PCa metastasis. Furthermore, loss of DAB2IP protein exhibits a strong clinical correlation, which supports the biologic function and clinical relevance of DAB2IP [19]. Taken together, loss of DAB2IP in PCa triggers the early event of PCa invasion and metastasis because DAB2IP is able to modulate multiple pathways involved in this event. Certainly, the assessment of DAB2IP in PCa specimens can be a valuable prognostic biomarker to predict the progression of PCa.

Loss of DAB2IP is Associated with Radiation and Chemotherapy Resistance of PCa Cells

The success of PSA screening has led to early diagnosis of localized PCa. Currently, the management of localized PCa ranges from active surveillance for indolent disease to therapeutic regimens such as radical prostatectomy, cryotherapy, high-intensity-focused ultrasound therapy, and internal or external radiation therapy. Radiation therapy has the advantage of being noninvasive and well tolerated. However, a significant proportion of high-risk patients will fail therapy and develop metastatic disease, for which no curative treatment currently exists [47]. Improving our understanding about biomarkers and their effect on therapeutic response may help us to develop personalized care. Indeed, our data have demonstrated that loss of DAB2IP in normal prostatic epithelial cells and PCa cells increase the resistance to ionizing radiation (IR) [48]. Furthermore, we found three potential mechanisms leading to the enhanced resistance to IR in DAB2IP-deficient PCa cells. First, it is due to faster DNA double-strand break (DSB) repair kinetics. Second, upon irradiation, DAB2IP-deficient cells enforced a robust G2/M cell cycle checkpoint. Third, DAB2IP-deficient cells showed resistance to IR-induced apoptosis that could result from a striking decrease in the expression levels of pro-apoptotic proteins caspase-3, caspase-8, and caspase-9, and significantly higher levels of anti-apoptotic proteins Bcl-2 and STAT3. Using DAB2IP-deficient cells, we have demonstrated a significant radiation dose enhancement using Epothilone B, a natural microtubule-stabilizing agent [49]. This radiosensitization can be attributed to delayed DSB repair, prolonged G_2 block, and increased apoptosis in cells entering the cell cycle after G_2/M arrest [50].

Chemotherapy is the standard of care for CRPC patients who have failed androgen deprivation therapy; however, a substantial proportion of men with CRPC do not benefit from docetaxel or other systemic regimens. Ultimately, those who do benefit invariably progress and die [51, 52]. The molecular mechanisms underlying the acquisition of chemoresistance by CRPC are not well defined. In our recent study, we unveiled a DAB2IP–clusterin signaling cascade leading to chemoresistance in PCa. Using a panel of DAB2IP-deficient cells, we observed that loss of DAB2IP could potentiate the chemoresistant cells to multiple therapeutic drugs with different mechanisms of action; these cells appear to exhibit an anti-apoptotic phenotype. On the other hand, restoring DAB2IP expression in these cells could significantly increase the cytoxicity of these agents. To further identify the underlying mechanism of multidrug resistance of DAB2IP-deficient cells, we were able to correlate the expression of secreted clusterin (sCLU), a crucial anti-apoptotic protein in CRPC, with the chemoresistance of these cells. In our study, DAB2IP is able to inhibit the expression of sCLU via transcriptional regulation. Furthermore, DAB2IP can inhibit the crosstalk between Wnt/β-catenin and IGF1/IGF1R signaling to initiate the early growth response-1 (Egr-1) transcription, which in turn induces sCLU expression (Fig. 10.2). Based on these observations, we conclude

that the assessment of DAB2IP status in clinical specimens could provide critical information for clinicians to select more appropriate regimens suitable for each individual patient to combat this disease.

The Role of DAB2IP in Modulating the Stemness of PCa

In general, metastatic CRPC exhibits many phenotypes similar to that of prostate stem cells (PSC) [53], suggesting that CRPC may be derived from cancer stem cell (CSC) population. Stem cells have self-renewal capacity, tremendous proliferative potential, but normally are quiescent, and can differentiate along one or several different cell lineages. In cancer stem cell theory, some cancer cells maintain typical stem cell properties, including self-renewal, differentiation, which are thought to be crucial for the initiation and maintenance of tumors as well as their metastases [54]. CSC is believed to play a critical role in tumor recurrence after conventional cancer therapies [55], because of its intrinsic nature of resistance to cell killing by chemicals and/or radiation via different mechanisms [56]. Thus, it will be important to define the regulation mechanisms of CSCs and develop alternative therapeutic strategies that are more specifically directed against CSCs. Hematopoietic stem cell studies have been the basis for identifying stem cell markers and isolating putative CSC from solid tumors including PCa [3, 13, 57]. Prostate CSCs have been identified by different groups based on (1) a panel of surface markers such as Sca-1 [58], CD44 [59, 60], and CD133 [61] corresponding to the stem cell markers of basal/stem cells from normal prostate, (2) their capability of forming prostaspheres in vitro [62, 63], and (3) their capability of initiating tumors in vivo [60, 64]. Although DAB2IP expression is associated with the normal basal cell population, little is known about the role of DAB2IP in regulating stem cells in the prostate. Our recent study demonstrated that loss of DAB2IP expression resulted in increased sphere-forming ability of PCa cells and stimulated their tumorigenic potential in vivo, indicating that DAB2IP is involved in the regulation of stemness of PCa. As mentioned, markers expressed by normal stem cells could also serve as markers of CSC, and some of them might have a functional role in maintaining stemness of CSC. Among these stem cell markers, CD117 was significantly increased in DAB2IP-deficient PCa cells. Restoring DAB2IP expression in these cells can suppress CD117 gene expression. By combining Chromatin immunoprecipitation and next generation DNA sequencing, we discovered a new distant silencer, located in the 3′-end of the CD117 gene, which can interact with DAB2IP. CD117, also known as proto-oncogene c-kit or stem cell factor (SCF or steel factor) receptor, is a tyrosine kinase receptor, plays a critical role in the development of the hematopoietic system [65], and has been demonstrated to be a marker for mouse prostate stem cells [66]. In our study, inhibition of CD117 in DAB2IP-deficient cells resulted in decreased stem cell properties, suggesting that DAB2IP-regulated stemness in PCa

is mediated through a CD117-elicited signal pathway. By enriching for a $CD117^{high}$ cell population from DAB2IP-deficient tumors, we found that $CD117^{high}$ has significant higher sphere-forming ability in vitro and tumor-initiating ability in vivo as compared with the $CD117^{low}$ population. We further delineated that signal cascade elicited by CD117 leading to CSC properties in which PI3K and mTOR pathways act as upstream effectors for c-myc activation and subsequent induction of EMT-activating factor ZEB1 (Fig. 10.2). Overall, DAB2IP is able to directly suppress CD117 gene transcription and inactivate CD117-elicited signal transduction by inhibiting PI3K.

Moreover, EMT, an initial step of cancer metastases, is considered to be a process of cell de-differentiation that could generate stem-like cells. Increasing evidence indicates that EMT plays a critical role not only in tumor metastasis but also in tumor recurrence and that it is tightly linked with the biology of CSCs [67–69]. However, despite efforts to link EMT with stem cell properties, the mechanisms by which EMT cells generate the cancer stem cells are not clear. We showed that loss of DAB2IP initiates EMT and facilitates PCa metastasis suggesting a role of DAB2IP in regulating CSC via EMT-mediated pathways [36]. In our study, loss of DAB2IP can lead to the significant increase of EMT-activator ZEB1 expression that is involved in increasing stem cell properties in PCa. ZEB1 has been shown to be critical for stemness maintenance by suppressing expression of stemness-inhibiting miRNAs (e.g., miR-200 family) [70]. This observation provides additional evidence for the mechanism of DAB2IP in balancing cell differentiation in prostatic epithelium.

The origins of CRPC are still controversial, because most cells in PCa tumors only express luminal cell markers such as CK8, 18. Therefore, it is believed that PCa is derived from the fully differentiated luminal cells [9, 10]. In contrast, CRPC may originate from the basal/stem cell population [71], because CRPC is androgen independent and expresses many basal cell-specific markers from metastatic sites. From our xenograft model, most of DAB2IP-positive tumors express luminal cell markers; however, DAB2IP-negative tumors express basal cell markers. The expression pattern of basal cell markers correlates with that of ZEB1 in DAB2IP-negative tumors. Consistent with this finding, ZEB1-expressing cells are located in the basal compartment of the prostate gland of $DAB2IP^{-/-}$ mice.

The phenotype of $DAB2IP^{-/-}$ mice provides good evidence of DAB2IP in maintaining prostatic epithelial homeostasis. For example, hyperplastic glands are observed after 6 months of age in prostate gland of $DAB2IP^{-/-}$ mice, which further supports the embryonic reawaking hypothesis. This hypothesis suggests that increases in the number of prostate stem cells, along with their progeny-transient amplifying cell population, is the initial development of prostatic intraepithelial hyperplasia or benign prostatic hyperplasia (BPH) [72]. Very likely, DAB2IP plays a critical role in this process.

On the other hand, we also found that loss of DAB2IP in immortalized normal human prostate epithelial cells (RWPE-1, PZ-HPV-7) could also enrich the stem cell population and induce cell transformation. By knocking down the endogenous DAB2IP, this increased $CD44^{high}/CD24^{low}$ cell population correlate with the

tumorigenicity of these cells, which supports a role for DAB2IP as a part of the homeostatic machinery in normal prostatic epithelial cells.

The Role of DAB2IP in Angiogenesis

Angiogenesis is the development of a new vascular network that provides tissues or cells with essential nutrients and oxygen and enables them to eliminate metabolic wastes [73]. This process is essential for embryogenesis, tissue repair, inflammatory diseases, and female reproductive cycle. Angiogenesis is a critical step for tumor growth and progression, which provides an additional blood supply that is necessary for the continuous expansion of primary tumor cells as well as for the spreading of metastatic tumor cells into distant organs [74]. Tumor angiogenesis involves the destruction of the extracellular matrix of preexisting blood vessels or insertion of interstitial tissue columns into the lumen of preexisting vessels, migration and proliferation of the endothelial cells, and eventually the formation of new endothelial tubes by the endothelial cells [75, 76]. It can be triggered by extracellular signals (such as growth factors) or by genetic alterations such as activation of oncogenes (e.g., PI3K) and/or mutations of tumor suppressor genes (e.g., PTEN and p53) [74, 75]. A number of factors such as hypoxia inducible factor (HIF) [77], fibroblast growth factor (FGF) [78], hepatocyte growth factor (HGF) [79], and members of the vascular endothelial growth factor (VEGF) family [80] have been identified to be associated with tumor angiogenesis during cancer progression. Especially, VEGF expression is markedly increased in PCa tissue compared to normal prostate tissues and is associated with microvessel density (MVD), tumor stage and grade, and disease-specific survival in patients with PCa [81].

We found that DAB2IP is an apoptosis-induced protein in endothelial cells [82]. DAB2IP$^{-/-}$ mice showed a significant increase of ischemia-induced arteriogenesis from existing vessels and neovascularization or vessel maturation. Moreover, angiogenesis and vessel maturation were increased in gastrocnemius muscles of DAB2IP$^{-/-}$ mice [83]. Consistently, in vitro DAB2IP overexpression significantly inhibited VEGF-induced endothelial cell (EC) migration and tube formation. Zhang et al. demonstrated that DAB2IP negatively regulates VEGFR2 signaling. VEGFR2 is the predominant receptor of VEGFs in angiogenic signaling and controls endothelial cell migration, proliferation, differentiation and survival, as well as vessel permeability and dilation [84]. Tyrosines 799 and 1173 of VEGFR2 are binding sites for the p85 subunit, resulting in increased PI3K and Akt activities in vitro [85, 86]. DAB2IP binds to the SH3 domain of the regulatory p85 subunit of PI3K through its PR motif, while it binds to a tyrosine-phosphorylated form of VEGFR2 through its C2 domain. This binding of DAB2IP to the VEGFR2–PI3K complex leads to an inhibition of VEGFR2-dependent angiogenic signaling [83].

Key angiogenic factors such as VEGF and interleukin-8 (IL-8) are also directly or indirectly enhanced by NF-κB activation [87, 88]. Min et al. [19] reported that ectopic DAB2IP expression suppresses NF-κB in endothelial cells, while loss of

DAB2IP promotes metastasis through NF-κB, functioning as signaling scaffolds that coordinately regulates NF-κB as well as Ras. Similar to a previous study [83], the expression of VEGF increased by NF-κB activation via DAB2IP shRNA. As VEGF is one of the most potent pro-angiogenic factors that can activate not only endothelial cells proliferation but also the blood vessel formation, these findings provide solid support for the role of DAB2IP in suppression of tumor angiogenesis.

There are several lines of evidence suggesting that PI3K-Akt signaling plays a major role in Ang-1 (one of the angiopoietins)-mediated cell migration, survival, and angiogenesis. The angiopoietins are a family of secreted proteins including three human angiopoietins such as Ang-1, Ang-2, and Ang-4, and one mouse angiopoietin, Ang-3. These angiopoietins promote angiogenesis and function by binding to their physiologic receptors, Tie-1 and Tie-2 [89]. Ang-1 induces phosphorylation of Tie2, its cell-surface receptor, which is then recruited and interacts with the p85 subunit of PI3K in a phosphotyrosine-dependent manner through Src homology 2 (SH2) domains, resulting in the induction of PI3K activities and activation of Akt [90] and Ang-1 induced survival, migration, and sprouting of endothelial cells through PI3K and Akt activation [90–92]. In vivo studies also showed that Ang-1 induces angiogenesis via increased Akt phosphorylation and PI3K-mediated endothelial nitric oxide synthase (eNOS) activation [93, 94]. As mentioned earlier, because DAB2IP can modulate PI3K and Akt activity by functioning as a scaffold protein, these results imply another role of DAB2IP in angiogenesis via regulation of Ang-1.

For the possible involvement of DAB2IP in tumor angiogenesis, two studies [95] have suggested that DAB2IP could be involved in tumor-associated angiogenesis and metastasis. For example, both VEGF and its receptor (VEGFRII) were elevated in hepatoma cells after endogenous *DAB2IP* gene expression was knocked down [95]. Similarly, increased VEGF mRNA was detected in prostatic epithelial cells transfected with shDAB2IP [19, 83]. These data suggest a role for the downregulation of DAB2IP in tumor cells on their surrounding microenvironment that is known to influence tumor behavior [96]. Nevertheless, the mechanism and functional role of DAB2IP in tumor angiogenesis require more detailed studies.

Conclusions

PCa is associated with altered signaling pathways leading to accelerated cell growth, prolonged cell survival, evading apoptosis, cancer invasion or metastasis, and drug resistance, and these pathways often converge into a signalosome. Based on data from us and other groups, DAB2IP is a unique protein functioning as a signalosome platform because its functional domains can recruit key effector molecules from different pathways and modulate their activities. Overall, in normal cell, the function of DAB2IP is to maintain the homeostasis of these pathways. Obviously, restoration of DAB2IP function in cancer cells becomes a desirable therapeutic strategy to prevent the onset of lethal PCa.

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Chapter 11
Tyrosine Kinases in Prostate Cancer

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Abstract Tyrosine kinases play essential roles in regulating cell proliferation and differentiation. Deregulation of the key tyrosine kinases governing these processes can lead to cellular transformation and is associated with tumor maintenance and progression. Amplification or dominant mutations of tyrosine kinases are not common in the pathogenesis of prostate cancer. However, studies from animal models and human prostate cancer specimens demonstrate that the level of tyrosine phosphorylation is elevated during prostate cancer progression. Numerous efforts have been devoted to identify the tyrosine kinases that are deregulated and responsible for such change during disease progression. A number of tyrosine kinases are shown to act as a driving force in disease progression and therefore may serve as potential drug targets. Currently, more than 20 different tyrosine kinase targets are under evaluation in drug discovery projects for cancer treatment. A number of tyrosine kinase inhibitors have been tested in various phases of clinical trials. This chapter provides an overview of identification, characterization, and targeting tyrosine kinases that are altered and critical in prostate cancer.

Identification of Tyrosine Kinases Deregulated in Prostate Cancer

Since the discovery of the first tyrosine kinase v-Src in 1979, 98 tyrosine kinases have been identified in the human genome. Although tyrosine phosphorylation represents <1 % of the entire phosphoproteome in a given cell, 50 % of tyrosine kinases

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in human genome are thought to contribute to human cancers [1, 2]. Identification of tyrosine kinases that play a role in driving prostate cancer progression may lead to development of new prognostic markers and targeted therapy. The first tyrosine kinase profile in prostate cancer was reported by Robinson et al. [3]. They used a degenerate RT-PCR-based method to identify about 40 different kinases expressed in a human prostate tumor xenograft CWR22. Since then, a variety of methods have been employed for the identification of novel protein tyrosine kinases (PTK), expression profiling in different tissues/disease states, quantification of kinase activity, and identification of interacting signaling proteins. These methods are categorized into the following subtypes.

Gene Expression Profiling

cDNA microarray technology allows the profiling of gene expression patterns in prostate tissues. A number of microarray analyses of human prostate tumors or xenograft tumors consistently showed that a number of EGF receptor family kinases are deregulated during prostate cancer progression [4–7]. In one study, hybridization-based cDNA microarrays was used to profile expression of 263 PTKs and other signaling proteins in 84 primary prostate cancer tissues and 7 xenografts. A molecular classification of prostate cancer composed of expression profiles of 66 genes, including a number of EGF receptor family kinases, was defined [4]. In another comparative gene expression profiling on human prostate tissues from tumors ($n=60$), adjacent normals ($n=63$) and cancer-free donors ($n=19$), c-SRC was identified as a gene upregulated in tumor tissues compared to normal donor tissues [8]. More recently, expression profiling has been performed in more defined cell population isolated by microdissection [9] and immune affinity purification [10]. In addition to cDNA microarrays, next-generation sequencing (NGS)-based platform has also been used to evaluate the spectrum of DNA/RNA alterations in formalin-fixed paraffin-embedded prostate cancer tissues [11].

Genomic DNA Arrays

Genomic instability is one of the major causes of oncogene amplification in human cancers. A genomic DNA microarray analysis of 57 oncogenes was performed on 38 paired hormone-naïve and hormone-resistant prostate cancer tissue samples [12]. Fifty-six percent of tumors were amplified for FGFR family kinases (FGFR1, 9 %; FGFR2, 47 %), 22 % of tumors for EGFR family kinases (EGFR, 11 %; HER2, 11 %), 19 % of tumors for the insulin receptor family kinases (INSR, 6 %; IGF1 receptor, 13 %), and 11 % for PDGFR family receptors and their ligands (PDGFRA, 8 %; PDGFB/c-sis, 3 %). Thus, over 70 % of prostate cancer cases showed amplification of classical growth factor receptor tyrosine kinases or their ligands.

The frequency of amplification of these genes appeared to not be associated with hormone resistance. However, a SRC-family non-receptor tyrosine kinase FGR/SRC-2 was amplified in significantly more hormone-resistant tumors (37 %) compared with hormone-sensitive tumors (0 %).

RNAi Screening

An siRNA library of 704 druggable kinases was used to knockdown kinases in the human prostate cancer cell line DU145 in an attempt to identify signaling molecules regulating expression of laminin binding glycans, a cell surface protein that is involved in cell attachment to basement membrane and inhibition of cell migration. Knockdown of ten genes significantly increased glycan expression. Among them, the non-receptor tyrosine kinase FER inhibited the expression of laminin-binding glycans via a STAT3-dependent manner. These results indicate that activation of the FER pathway impairs basement attachment and increases tumor cell migration by negatively controlling the expression of genes required to synthesize laminin-binding glycans [13]. In another recent study to identify signaling pathways that regulate prostate cancer cell growth, a screen using a panel of lentiviral-based shRNAs targeting 673 human kinases was performed in the prostate cancer cell line LNCaP. Knockdown of 46 kinases in LNCaP inhibited cell growth [14]. In addition to the top six hits validated in this report, knocking down a number of tyrosine kinases attenuated proliferation of LNCaP cells (personal communication with Dr. Daniel Gioeli at University of Virginia). Further evaluation of these kinase targets may lay the foundation for developing more effective treatments for castration-resistant prostate cancer (CRPC).

Phosphotyrosine Proteomics

Phosphotyrosine proteomics is a widely used high throughput method to study global phosphorylated substrate profiles of tyrosine kinases in cancer. It usually involves two steps (1) enrichment of tyrosine phosphorylated peptides by immunoaffinity purification using a phosphor-specific antibody from a proteolytic digest, followed by (2) LC-MS/MS-based peptide identification to locate the phosphorylated residue within a sequence. Stable isotope labeling by amino acids in cell culture (SILAC) can be used for in vivo/vitro incorporation of a label into proteins before MS-based quantitative proteomics [15]. Alternatively, post-proteolysis, chemical labeling (e.g., dimethylation) and isobaric tags for relative and absolute quantitation (iTRAQ) can be applied [15]. This approach has been used to identify more than 1,000 nonredundant phosphotyrosine peptides in <6 h of MS analysis [16]. The high sensitivity and specificity of the technique has made it one of the frequently used for global phosphotyrosine mapping. Phosphopreoteomics have

been performed to identify substrates of a particular tyrosine kinase (e.g., c-SRC). This strategy led to the identification of 213 phosphoproteins of which 29 c-SRC substrate proteins were considered to be novel [17]. A mouse model mimicking prostate cancer progression after overexpression of commonly perturbed non-tyrosine kinase oncogenes (e.g., ERG, KRAS) in combination with AKT led to increased tyrosine phosphorylation at the carcinoma stage. Phosphotyrosine peptide enrichment followed by quantitative mass spectrometry revealed oncogene-specific tyrosine kinase profiles, e.g., activation of EGFR, Ephrin type-A receptor 2 (EPHA2), JAK2, ABL1, and SRC [18].

Protein Microarrays

The major proteins recruited to tyrosine phosphorylated residues are those containing SH2 domains. Putative Src homology-2 (SH2) domain proteins can be immobilized on membranes. Cell lysates are applied to these membranes, and phosphorylated proteins are allowed to interact with the immobilized proteins. Bound proteins are measured using a chemiluminescence-based detection system [19]. Conversely, in the far-Western filter binding-based method, the protein extract is immobilized by first separating the proteins on a 1D gel and then transferring to a nitrocellulose or PVDF membrane. The membrane is probed with labeled GST-SH2 fusion proteins [20]. In addition, peptide microarrays are amenable to high throughput by using automation like microarray spotters and scanners. High density arrays are prepared by spotting substrate peptides on premodified high-amino terminal glass slides. Cell lysates are applied, and phosphorylation events are detected using fluorescence-labeled antiphosphotyrosine antibody [21]. Protein microarrays have been utilized for identification of serum biomarkers for prostate cancer. This is based on the premise that xenograft-bearing nude mice (nu/nu Balb/c) have antigens released by the xenografts into serum. The immuno-competent strain with the same background as the nude mice (nu/+ Balb/c) is then immunized with the serum from these nude mice to generate an antibody response. A protein microarray is probed with the serum from these immunized mice. Several prostate cancer-derived antigens were identified and validated in serum of prostate cancer patients. Some of the identified biomarker proteins were members of the TAM receptor family TYRO3, AXL, and MER [22].

Tissue Microarrays

A prostate tissue microarray (TMA) can be used to evaluate the extent of tyrosine phosphorylation in human prostate tissue samples of different tumor grades by immunohistochemistry. A recent survey of a TMA containing 75 prostatectomy specimens comprising areas of normal prostate, high-grade prostatic intraepithelial neoplasia (HGPIN), and hormone-naïve prostate cancer (HNPC) were performed

using a monoclonal anti-phosphotyrosine antibody 4G10 [18]. The level of tyrosine phosphorylation in normal and hormone-naïve tissue samples was very low and might well be negligible compared to that in castration-resistant prostate cancer (CRPC) tissues. These observations are consistent with a previous study of xenograft tumor models showing a robust increase of tyrosine phosphorylation in hormone-resistant tumor tissues detected by Western blot [23]. These studies strongly support a role of tyrosine kinases in CRPC. It is possible that tumor cells utilize tyrosine kinases activated by autocrine/paracrine factors to compensate for the loss of androgens.

Other Methods

A number of other methods have been used to quantify tyrosine kinase expression or activity via detecting protein–protein interactions and receptor tyrosine kinase (RTK) conformational changes. An antibody-based time-resolved Forster resonance energy transfer (TR-FRET) was developed to quantify EGFR and HER2 expression as well as their activation through HER homo- or heterodimer formation in tumor cyrosections [24]. Bioluminescence resonance energy transfer (BRET) has been used to monitor homodimeric insulin/IGF-1 or heterodimeric insulin/IGF-1 conformational changes following application of ligands [25]. An electrochemical method has been developed to measure the activity of PTKs. A PTK substrate Poly(Glu/Tyr) peptide was immobilized on the surface of indium tin oxide (ITO) electrode. The tyrosine (Tyr) in the polypeptide was used as an electrochemical sensor. Phosphorylation of the Tyr residue led to a loss of its electrochemical current, thus providing a sensing mechanism for PTK activity. This assay can be used as a high throughput screen for measuring kinase activity besides screening of potential kinase inhibitors [26]. In another variation of the above method, the transfer of $^{33}PO_4$ from ATP to the synthetic substrate poly(Glu/Tyr) peptide attached to a bioactive surface of a scintillating microtiter plates was used to evaluate PTK activity [27]. In another novel detection method, an array-based surface plasmon resonance (SPR) apparatus has been used to gauge the extent of phosphorylation. Recombinant substrate proteins with FLAG/GST tags were captured by a corresponding capture antibody on a sensor chip. Cell lysates were added to allow for phosphorylation of tyrosine residues. The phospho-tyrosine residues were detected by an anti-pTyr antibody. The extent of binding of the anti pTyr antibody was detected by SPR. A throughput of 1,000 samples/day can be achieved using this method [28].

Pathway Signature

The development of prostate cancer is a complex process involving deregulation of multiple cell signaling pathways central to the control of cell growth and cell fate. Gene expression signatures can be used for cancer subtype classification,

monitoring disease recurrence, and prediction of patient response to specific therapies [29, 30]. Gene expression signatures can be identified through analyzing a large collection of human cancers to determine the activation status of tyrosine kinase and other oncogenic pathways. A study on gene expression profile datasets derived from 250 human prostate cancer patients showed that a significant fraction (~5 %) of genes upregulated experimentally by c-SRC and HER2 were co-expressed in human tumors with the oncogene corresponding to the pathway signature [31]. Predictions of pathway deregulation in cancer cell lines are able to predict the sensitivity to therapeutic agents that target components of the pathway [32]. Therefore, linking pathway deregulation with sensitivity to therapeutics that target components of the pathway makes it possible to guide more effective targeted therapeutics based on these experimentally derived oncogenic pathway signatures.

Role of Tyrosine Kinases in Prostate Cancer: Studies from Model Systems

Signaling pathways mediated by tyrosine kinases play an important role in maintenance of homeostasis of normal prostate glands as well as pathogenesis of prostate cancer. It was reported in an early study in 1996 that a polyclonal anti-phosphotyrosine antibody preferentially stains the basal cells in normal dog prostates, and the staining intensity was associated with cell proliferation [33]. The preferential localization of phosphotyrosine proteins in basal cells, the increase of the level per cell, and the number of positive cells in androgen-treated prostates from castrated dogs suggest that prostate regeneration is likely to involve growth factor regulated signal transduction pathways. In addition, the number of positive basal cells and their staining intensity were increased in naturally occurred hyperplastic lesions in dog prostates. These observations suggest an intimate link between cell proliferation and activation of tyrosine kinase pathways in prostate glands. Several independent studies demonstrated that the level of tyrosine phosphorylation is elevated in CRPC xenograft tissues compared to hormone-naïve counterparts [23, 34]. Subsequently, similar change in human CRPC tissues was reported [18]. Currently, our understanding of the role of tyrosine kinases in prostate cancer is largely derived from studies in various model systems and subsequent validation in human tissue samples. The following is a summary of some important studies on tyrosine kinase families that are deregulated in prostate cancer.

Non-receptor Tyrosine Kinases in Prostate Cancer

SRC Family Kinases

SRC family kinases represent one of the largest families of non-receptor tyrosine kinase, comprising nine members viz. BLK, FGR, FYN, HCK, LCK, LYN, SRC,

YES, and YRK. In addition, PTK6 (a.k.a Brk or Sik) is also considered to be related to SRC. They constitute the second tier of signal transduction molecules that collate signals from different cell surface receptors (growth factor receptors, adhesion and integrin receptors, GPCRs, immunological recognition receptors, and ion channels) and transduce them to downstream molecules to affect various cellular activities like differentiation, cell-cycle progression, adhesion, and migration. SRC is a 60 kDa protein consisting of different SRC homology domains (SH). Towards the amino terminal region is the SH4 domain, which has signals for lipid modifications along with a highly nonconserved region specific for each SFK member thought to be responsible for its unique protein–protein interactions. This domain is linked to the SH3 and SH2 domains, which regulate the activity of the kinase besides participating in protein interactions. The C-terminal SH1 domain is the kinase domain with a short carboxyl terminal containing the Y530 residue (Y527 in chicken Src) which when phosphorylated leads to the closed conformation of the kinase [35]. The phosporylation status of SRC is maintained by C-terminal SRC kinase (CSK) and CSK homologous kinase. SRC can be activated by any of these three ways: binding of ligand-bound cell surface receptors, binding of cytosolic proteins such as focal adhesion kinase (FAK), or Y530 dephosphorylation by several cellular phosphatases.

SRC transduces signals by phosphorylating substrates in the cytosol, inner face of the plasma membrane, cell–matrix, cell–cell adhesions, or by providing phosphotyrosyl residue docking sites on its SH2 domain [36]. The SFK members SRC and LYN have been implicated in the pathogenesis of prostate cancer. Elevated SFK activity in tumors from prostate cancer patients is associated with shorter responses to androgen-ablation therapy, metastasis to the bone, and shorter survival [37]. SRC and LYN are highly expressed in prostate cancer cell lines and tissue specimens [38, 39]. SRC appears to be a ubiquitous transducer having a role in androgen-dependent and androgen-independent AR activation, growth factor receptor (EGF-R)-mediated cell proliferation, migration and invasion, receptor tyrosine kinase (IGF-1)-mediated cancer progression, and intracellular protein (VEGF/FAK)-mediated angiogenesis, migration, and invasion. Most of the signals are transmitted through the SRC/FAK/ETK tyrosine kinase complex [40].

Androgen or estrogen treatment of LNCaP cells leads to a simultaneous association with androgen receptor (AR) and estrogen receptor beta (ER-beta) via interactions of the SRC SH3 domain with a proline-rich stretch of AR and the SRC SH2 domain with phosphotyrosine 537 of ER-beta, respectively, thereby activating the Src/Raf-1/Erk-2 pathway leading to cell proliferation [41]. SRC has also been implicated in the androgen-independent growth of prostate cancer cells. IL-8 led to androgen-independent growth and migration of LNCaP cells via activation of the SRC, FAK, and ERK kinases [42]. Neurotrophic factors bombensin and neurotensin induced LNCaP cell growth in the absence of androgen through activation of the AR. The connecting link between bombensin bound to its GPCR and AR were the three nonreceptor tyrosine kinases viz. focal adhesion kinase (FAK), SRC, and ETK/BMX [43]. c-SRC also participates in signaling events mediated by a variety of polypeptide growth factor receptors, including the epidermal growth factor receptor (EGFR), platelet derived growth factor

receptor (PDGFR), and insulin-like growth factor 1 (IGF-1). Elevated levels of c-SRC and EGFR are found in a number of human cancers including prostate cancer. c-SRC and EGFR interact and may contribute to aggressive phenotypes in multiple human cancers [44, 45]. SRC mediates EGF-induced phosphorylation of AR Tyr534 and elevated SRC kinase activity is detected in CRPC tissues [23]. SFK can also associate with PDGFR via their SH2 domain docked onto the phosphotyrosine 579 of PDGFR [46]. This association leads to the activation of SFK by releasing the intramolecular interaction between the SH2 domain and the cytoplasmic tail. Hypoxia leads to activation of SRC, thereby inducing VEGF expression in the highly metastatic prostate cancer cell line PC3 [47]. Androgens upregulated the expression of IGF-1R and sensitized cells to the mitogenic and motogenic effects of IGF-1 through activation of the SRC–ERK pathway in LNCaP cells [48]. EphA2 expression correlates with the metastatic potential of the prostate cancer cell lines [49], and SRC has a role in upregulation of EphA2 in noncancerous tissues [50], thus, providing a potential link between the two kinases. Focal adhesion kinase (FAK) plays a key role in regulation of cell migration. FAK expression is higher in highly metastatic prostate cancer cell lines DU145 and PC3 compared to LNCaP. The integrin-stimulated FAK/SRC signal transduction pathway appears to be essential for migration of prostate cancer cell lines. Inhibiting SFK activity using a small molecule inhibitor significantly reduced migration of prostate cancer cells [51]. A murine prostate cancer cell line, generated via selective transduction with v-Src oncogene, developed lung metastasis in immune competent mice and demonstrated oncogene-specific molecular signatures that were recapitulated in human prostate cancer [32]. SRC also has key roles in regulating osteoclast function and in the pathogenesis of bone metastases. Bone metastasis occurs in about 70 % of patients with advanced prostate cancer [52]. In normal bone, SRC plays an active role in bone turnover through osteoclastic activity. SRC kinase activity and the SRC-SH2 domain-dependent SRC/PYK2 complex are required for bone resorption. Src-dependent phosphorylation of CBL on Tyr-731 was found to be essential for recruitment of PI3K to cell membrane for optimum osteoclastic activity [53].

LYN is expressed in normal prostate epithelia, in 95 % primary human prostate cancer, and in all prostate cancer cell lines tested [39]. Lyn knockout mice displayed abnormal prostate morphogenesis, thus, underscoring the importance of Lyn in normal prostate development. A peptide inhibitor of LYN, KRX123, reduced cell proliferation of three hormone refractory prostate cancer cell lines viz. PC3, DU145, and TSU-Pr1 [39]. In a prostate regeneration in vivo model, ectopic expression of constitutively activated SRC, FYN, and LYN kinases exhibited differential capacities for transformation of prostate epithelium, with SRC kinase representing the strongest oncogenic phenotype, followed by FYN and then LYN [54]. SRC, LYN, and FGR expression and activation is increased in the transition from androgen sensitive prostate cancer to castration-resistant prostate cancer [37]. Dasatinib, an SFK inhibitor, blocked the kinase activity of both SRC and LYN and reduced proliferation, migration, and invasion. In vivo in an orthotopic nude mouse model,

Dasatinib reduced cellular proliferation as measured by immunohistochemistry of proliferating cellular nuclear antigen (PCNA). SiRNA targeting Lyn reduced cell proliferation and siRNA targeting Src reduced migration and tumor growth under androgen-depleted conditions [23, 55].

BRK/PTK6 is a non-receptor tyrosine kinase related to SFK. Its localization is nuclear in the more differentiated cell line LNCaP and is cytoplasmic in the androgen-independent PC3 cell line [56]. Cytoplasmic localization of BRK is associated with cell proliferation and migration whereas nuclear localization reduced proliferation and migration [57]. BRK/PTK6 has also been implicated in migration and invasion through the formation of peripheral adhesion complexes by phosphorylation of p130-CRK-associated substrate (p130CAS) [58].

TEC Family Kinases

The TEC family non-receptor tyrosine kinases include four members viz. TEC, BTK, ITK, and BMX/ETK. Structurally, they are similar to SRC family kinases with an exception that they all have one plekstrin homology (PH) domain at their N-termini. Expression of ETK (a.k.a BMX) in prostate cancer cells was first reported in an unbiased kinase profiling [3]. Subsequent studies showed that ETK is a downstream effector of PI3-kinase and plays a role in IL6 induced neuroendocrine differentiation of LNCaP cells [59]. The PI3-kinase/ETK pathway protected LNCaP cells from apoptosis on treatment with either photodynamic therapy or thapsigargin [60]. ETK can also interact with tumor suppressor p53 in the cytoplasm through its SH3 domain and the proline-rich domain of p53. This interaction leads to a bidirectional reduction in activity of both the proteins. Upon DNA damage, activation of p53 leads to a downregulation of ETK thereby inducing apoptosis. Over-expression of ETK on the other hand leads to the transcriptional repression of p53 thereby preventing its interaction with mitochondrial protein BAK and conferring resistance to apoptosis by DNA damaging agents like doxorubicin [61]. Over-expression of ETK in mouse prostate epithelium resulted in changes resembling prostatic intraepithelial neoplasia (PIN) lesions thus underscoring its role in prostate cancer development. In the ETK transgenic prostate, an increase in luminal epithelial cell proliferation was observed which could in part be ascribed to AKT and STAT3 activation. ETK may also have a role in chromatin remodeling through modulating the activity of acetyltransferase CBP [62]. Furthermore, ETK is upregulated in both mouse and human prostate tissues in response to androgen ablation [63]. Overexpression of ETK induces AR Y534 phosphorylation like SRC kinase and stabilizes AR protein by interacting with AR through its SH2 domain and preventing the association of AR with Mdm2 thereby stabilizing AR under androgen-deficient conditions. Knocking down ETK expression in prostate cancer cell lines attenuated cell proliferation under androgen-depleted conditions. This suggests an important role of ETK in CRPC, and ETK may serve as a potential therapeutic target.

FAK Family Kinases

FAK expression is higher in highly metastatic prostate cancer cell lines (DU145 and PC3) as compared to the less metastatic cell line (LNCaP) [51]. The role of FAK in prostate cancer is primarily in cell motility and cytoskeletal rearrangement. Members of the EGFR family, especially ErbB2, have been implicated in conferring hormone resistance in prostate cancer as well as in oncogenic transformation and cell invasion. Focal adhesion kinase (FAK) is essential for the activity of ErbB2 receptor tyrosine kinases [64]. A bone morphogenetic protein (BMP) called GDF-9 increased cell adhesion and motility of PC3 cells through activation of FAK and paxillin [65]. Elevated levels of macrophage inhibitory cytokine-1(MIC-1) are found in the sera of metastatic prostate cancer compared with benign or healthy adults. Overexpression of MIC-1 enhanced metastatic potential through changes in actin organization. These changes were mediated by increased phosphorylation of FAK- and GTP-bound RhoA [66]. Endothelin-1-mediated migration of prostate cancer cells was also found to be regulated by FAK [67]. In addition, FAK plays an important role in VEGF-mediated angiogenesis. VEGF activates FAK, translocating it to the cell–cell junction where it binds to the vascular endothelial cadherin (VE-cadherin) cytoplasmic tail and phosphorylates β-catenin on Y142, facilitating VE-cadherin-β-catenin dissociation thereby leading to endothelial cell junction breakdown [68].

Other Non-receptor Tyrosine Kinases

The CDC42-associated tyrosine kinase ACK1 is upregulated in CRPCs and play a critical role in heregulin-induced activation of AR by phosphorylating AR at Y267 [34]. Another non-receptor tyrosine kinase FER inhibits basement attachment and increases tumor cell migration by negatively controlling the expression of genes required to synthesize laminin-binding glycans [13].

Receptor Tyrosine Kinases in Prostate Cancer

EGFR/ErbB Family Kinases

The ErbB (erythroblastic leukemia viral oncogene homolog) or EGF (epidermal growth factor) family of transmembrane RTKs plays an important role during the growth and development of a number of organs including the heart, the mammary gland, and the central nervous system. ErbB overexpression is associated with tumorigenesis of the breast, prostate gland, ovaries, and brain. The ErbB family includes four members, EGFR/ErbB1, ErbB2, ErbB3, and HerbB4. All ErbBs have an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic kinase domain [69]. ErbB1, ErbB3, and ErbB4 can homo/

heterodimerize, but ErbB2 is an orphan receptor that heterodimerizes with other ErbB family members. ErbB2 and ErbB3 immunostaining is different in benign vs. prostatic intraepithelial neoplasia (PIN) lesions. In the benign glands, immunostaining for both ErbB2 and ErbB3 was strongest in the basal cells and absent or weak in the luminal cells. Both the proteins within luminal cells were localized at the cell membranes. In the PIN lesions, the basal cells demonstrated strong to moderate immunostaining as in benign prostate. However, in contrast to benign glands, moderate to strong immunostaining for ErbB2 and ErbB3 was observed within the cytoplasm and cell membranes of the prostatic intraepithelial neoplastic luminal cells. Thus, ErbB2 and ErbB3 might have a role in early development of prostate cancer [70]. ErbB2 receptor expression was detected by immunohistochemistry in prostate cancer tissue specimens especially in samples progressing from an androgen-dependent to an androgen-independent state. In the absence of androgen, there is cross talk between the EGFR family receptors and the AR with MAPK and PI3-kinase transduction pathways playing a role [71]. HER2/ErbB2 could activate AR under low androgen conditions through activation of the MAPK pathway and interaction with AR coactivators like ARA70 [72]. SRC and EGFR are upregulated in a number of cancers. When SRC and EGFR were overexpressed, there was increased DNA synthesis, growth in soft agar, and tumor formation in nude mice. This was associated with a heterocomplex formation between SRC and activated EGFR [44].

TAM Family of Kinases

AXL belongs to the TAM (TYRO3, AXL, and MER) family of receptor tyrosine kinases. These receptors are distinguished from each other by a conserved sequence in the kinase domain and adhesion molecule-like domains in the extracellular region. Activation of AXL occurs upon binding to the growth arrest specific gene 6 (GAS6). AXL is involved in cell survival, cell adhesion, chemotaxis, and blood vessel formation [73]. Androgen-independent cell lines PC3 and DU145 express high levels of AXL compared to androgen-sensitive LNCaP cell line [73]. AXL is overexpressed in a number of cancers and is associated with invasiveness, angiogenesis, and metastasis [74]. AXL was found to be upregulated in 50 % of adenocarcinomas as compared to normal prostates [73]. Inhibition of AXL leads to reduction in proliferation, invasion, migration, and tumor growth. siRNA knockdown of AXL lead to transcription of cell survival genes related to NFkB [73]. AXL plays a role in the epithelial–mesenchymal transitioning in prostate cancer. Knockdown of AXL in PC3 and DU145 cells resulted in decreased expression of mesenchymal markers including SNAIL, SLUG, and N-cadherin, and enhanced expression of the epithelial marker E-cadherin. Knockdown of AXL in PC3 and DU145 cells resulted in decreased migration and invasion in vitro. Immunochemical staining of human prostate cancer TMAs showed that AXL, GAS6, and hypoxia-inducible factor-1alpha (HIF-1alpha; indicator of hypoxia) were coexpressed in prostate cancer and bone metastases compared with normal tissues [75]. AXL also has a role in vascular

permeability and angiogenesis by mediating VEGF signaling through activation of the PI3K/AKT pathway [76].

Eph and Ephrin RTKs

Eph (erythropoietin producing hepatoma) and Eprhins constitute the largest family of RTK, which consists of nine A type Ephs (EphA1–8 and A10), five A type Ephrins (Ephrin A1–5), five B-type Ephs (Eph 1–4 and B6), and three B type Eprhins (EphrinB1–3). Generally, EphAs bind to Ephrin As and EphBs bind to Ephrin Bs; however, there are a few exceptions [77]. Expression of EphA2 correlates with the metastatic potential of prostate cancer cell lines viz; low levels in LNCaP, intermediate levels in DU-145 and high levels in PC3 cells [49]. EphA2 expression was upregulated in human metastatic prostate tissues compared to benign prostate [49]. EphB4 is upregulated in prostate cancer cell lines PC3, DU145, and LNCaP. Moreover, EphB4 expression was higher in prostate cancer tissues when compared to benign tissues [78]. EphA2 has ligand-dependent tumor enhancing or suppressing roles in cancer. Upon activation by oncogenic pathways, it acts as a tumor enhancer whereas it acts as a tumor suppressor when activated by EphA1. Ephrin A1 ligand-dependent activation of EphA2 decreased the growth of PC3 cells and inhibited AKT-mTORC1 pathway, which is hyperactivated due to the loss of the PTEN tumor suppressor phosphatase [79]. EphA2 and EphA4 activation by Ephrin-As on adjacent prostate cancer cells triggers repulsion of two colliding cancer cells through RhoA activation, while activation of EphB3 and EphB4 on prostate cancer cells by stromal Ephrin-B2 stimulates invasive migration of cancer cells through Cdc42 activation. These processes enhance cancer cells scattering from the primary tumor mass and promote migration and invasion through the stroma [80].

Platelet-Derived Growth Factor Receptor

Platelet-derived growth factor (PDGF) was one of the first polypeptide growth factors identified that signals through a cell surface tyrosine kinase receptor (PDGFR) to stimulate various cellular processes including growth, proliferation, and differentiation. EMT can be triggered by various growth factors such as platelet-derived growth factor-D (PDGF-D). PDGF-D acts through its cognate receptor PDGFR-β. Significant downregulation of miR-200 family in PC3 PDGF-D cells as well as in PC3 cells exposed to purified active PDGF-D protein, resulted in the EMT phenotype through the upregulation of ZEB1, ZEB2, and snail2 expression (mesenchymal markers). Moreover, transfection of PC3 PDGF-D cells with miR-200b lead to reduced cell migration, invasion, and cell detachment. Thus PDGF-D through activation of PDGFR-β has a role in EMT transition [81]. In a prostate-specific PTEN deletion mouse model, there was an increase in PDGFRβ and its ligand PDGF-D expression. Thus, loss of PTEN might lead to tumor progression via PDGFRβ signal transduction [82]. PDGF acts as a survival factor in prostate cancer via

upregulation of myeloid cell leukemia-1 (MCL-1). Upon PDGF stimulation, β-catenin translocates to the nucleus and forms a transcriptional complex by binding to hypoxia inducible factor-1α (HIF-1α) at the MCL-1 promoter [83]. PDGF-D was found to be more effective in tumor cell migration and invasion as compared to PDGF-B in vitro. In vivo PDGF-D supported tumoigenesis and angiogenesis more effectively than PDGF-B [84].

Insulin-Like Growth Factor-1 Receptor

The insulin-like growth factor-1 receptor (IGF-1R) is widely expressed in many cell types and regulates a variety of cellular responses including proliferation, and the protection of cells from programmed cell death or apoptosis. Forced over-expression of IGF-1R results in the malignant transformation of cultured cells. Inhibition of IGF-1R expression can reverse the transformed phenotype of tumor cells and promote apoptosis in vivo. The IGF-1 axis is a predisposing factor in the pathogenesis of human prostate cancer. Thus, IGF-1R is a potential therapeutic target [85]. In LNCaP cells, androgens upregulate the expression of IGF-1R and sensitize cells to the mitogenic and motogenic effects of IGF-1 through activation of the SRC–ERK pathway [48]. Action of insulin-like growth factor is modulated by its binding proteins IGFBPs. Expression of IGFBP-5 is significantly increased in normal and malignant prostate tissue after androgen withdrawal. Increased IGFBP-5 expression seems to be an adaptive response to castration that promotes the anti-apoptotic and mitogenic effects of IGF-1 thereby leading to androgen independence [86].

Hepatocyte Growth Factor Receptor

The proto-oncogene receptor tyrosine kinase c-MET encodes the high-affinity receptor for hepatocyte growth factor (HGF, a.k.a scatter factor). Deregulation of the HGF-c-Met pathway plays a significant oncogenic role in many cancers. The growth-stimulating effects of HGF on various tumor cells were first observed in hepatectomized nude mice, in which accelerated tumor growth directly coincided with liver regeneration [87]. A study on the comparison of biomarker expression in benign prostatic epithelium and intraepithelial neoplasia (PIN) showed that the strong expression of c-MET is restricted to basal cells within the benign epithelium but becomes detectable in luminal cells of PIN lesions [88]. Therefore, c-MET-mediated signaling driven by autocrine/paracrine factors in the prostate may play a role in initiation of prostate cancer. The potential important role of HGF and its receptor c-MET in prostate cancer progression was first appreciated in a study on metastatic LNCaP derivative cell lines [89]. A later study of human prostate tissues showed that c-MET protein was detectable in 84 % of primary prostate cancer samples and 100 % of lymph node and bone metastases, whereas only 18 % of benign prostate hyperplasia samples expressed c-MET [90]. This is supported by another independent study also showing high expression of c-MET in bone metastasis [91].

The action of HGF appears to switch from paracrine to autocrine during progression to a castration-resistant state, as shown in an androgen-sensitive prostate cancer xenograft model CWR22 and its castration-resistant derivative CWR22R [92]. CWR22 tumor cells express c-MET but not HSF, while CWR22R tumor cells express both. Expression of HSF and c-MET in human prostate cancer tissues was detected by immunohistochemistry, in situ hybridization, and RT-PCR analysis [93]. It is notable that differential localization of c-MET was observed in tumors at various grades. In low grade tumors, c-MET is mainly at the plasma membrane while in higher grade tumors, more cytoplasmic staining is detected. The results from these studies suggest that HGF is involved in growth of human prostate cancer and that progression from the androgen-dependent to the castration-resistant state is associated with an adaptive switch in its support mechanism from paracrine to autocrine. In addition, expression of c-MET in prostate cancer cells is repressed by androgen receptor (AR) [94]. Expression of c-MET is upregulated by androgen deprivation, and c-MET appears to be preferentially expressed on androgen-insensitive, metastatic cells [95]. Compensatory upregulation of c-MET expression in prostate cancer cells in response to androgen withdrawal or knockdown of AR using specific small interfering RNA suggest that c-Met signaling is utilized for survival and growth following androgen depletion [96]. Therefore, blockade of the activation of the HGF/c-Met pathway may be of therapeutic benefit when combined with androgen ablation.

HGF treatment promotes the association of the E-cadherin/catenin complex with c-MET in LNCaP cells, thereby regulating intercellular adhesion in prostate cancer [97]. HGF increases the invasive potential of prostate cancer cells, probably through enhancement of cell motility and the production of matrix metalloproteinases, urokinase-type plasminogen activator (u-PA), and its receptor (uPAR) [98]. RNA interference revealed that ligand-independent MET activity is required for tumor cell signaling and survival [99]. Activation of c-MET has been shown to protect cancer cells against the DNA-damaging agent adriamycin in a SRC-dependent manner [100]. c-MET is physically associated with several TRAIL receptors and regulates their protein stability thereby promoting TRAIL resistance in tumor cells [101]. Furthermore, c-MET distribution is significantly different between patients with metastatic cancer and patients with only localize prostate cancer and normal individuals without cancer. c-MET protein is detectable in patients with metastatic prostate cancer. Therefore, urinary c-MET may provide a noninvasive biomarker indicative of metastatic prostate cancer [102].

Targeting Tyrosine Kinases in Prostate Cancer

Due to unprecedented progress made in the past decades in understanding the human kinome, increasing numbers of receptor and non-receptor tyrosine kinases have been identified as valuable molecular targets for cancer prevention and

treatment. Inhibition of oncogenic tyrosine kinases provides new targeted therapies for cancer. The isoflavone genistein, which belongs to a large family of flavonoids synthesized by plants and is a naturally occurring pan tyrosine kinase inhibitor (TKI), was discovered in soy [103]. Epidemiological reports suggest that Asians consuming a diet high in soy have a low incidence of prostate cancer. Soy and genistein suppress the development of prostate cancer in various animal models. A recent large survey on 920 African–American men and 977 European–American men showed that men with the highest total intake of flavonoids had a 25 % lower risk for aggressive prostate cancer compared to those with the lowest flavonoid intake. Although the role of flavonoids in cancer prevention is well documented, it is unclear whether the anticancer activity of flavonoids is solely due to inhibition of tyrosine kinases and whether flavonoids can be used as an effective treatment for prostate cancer, especially for advanced metastatic prostate cancer. Currently, more than 20 different tyrosine kinase targets are under evaluation in drug discovery projects in oncology. A number of more selective TKIs are in various stages of clinical trials, but none of them have been approved yet by the FDA for treatment of prostate cancer (Table 11.1). These inhibitors are either small molecules or antibodies. VEGFR antagonists and SRC kinase inhibitors represent two target classes with molecules in advanced clinical trials (Phase III) compared to other targets. Herein, we will review the current state of development of targeted therapeutics against tyrosine kinases in prostate cancer.

SFK Inhibitors

Dasatinib

Dasatinib (BMS-354825, Sprycel) is a small-molecule ATP-competitive multi-targeted kinase inhibitor of BCR-ABL and SFKs. Dasatinib also inhibits the activity of other kinases such as TEC family kinases, ACK1, and some RTKs (c-KIT, c-FMS, PDGFR, DDR1, and Ephrin receptors) [104, 105]. Dasatinib is approved by the FDA for the treatment of imatinib-resistant CML. Dasatinib successfully reduced proliferation, adhesion, migration, and invasion of prostate cancer cells in xenograft models, reducing tumor mass and decreasing metastatic dissemination of tumor cells [55]. Dasatinib also acts on the tumor microenvironment, particularly in bone, where it inhibits osteoclastic activity and promotes osteogenesis. Several preclinical studies have shown that Dasatinib potentiates the anticancer activity of several FDA approved drugs for cancer treatment [106]. The efficacy of Dasatinib as a monotherapy for patients with advanced prostate cancer is modest. It is currently being investigated in combination with docetaxel and predinisone. A remarkable improvement in bone metastasized prostate cancer has been shown [107]. Dasatinib is currently the most advanced small molecule inhibitor for SFKs that has been tested in Phase III trials for prostate cancer therapy.

Table 11.1 TKIs under clinical evaluation for treatment of prostate cancer

Target	Agent	Phase	Aim	Primary end point (Identifier)
SRC	Dasatinib	III	Determine survival benefit in patients with CRPC who receive Dasatinib in addition to Docetaxel and Prednisone	Overall survival (NCT00744497)
VEGF	Aflibercept	III	Improvement in overall survival compared to prednisolone/docetaxel	Overall survival (NCT00519285)
Other compounds in early clinical trials representing different tyrosine kinases as targets				
PDGFR	Imatinib	I/II	Docetaxel and Imatinib in HRPC	NCT00861471 (study suspended due to slow accrual)
EGFR	Gefitinib	I/II	To study effect of Gefitinib concurrent with radiotherapy in patients with non-metastatic prostate cancer	NCT00239291
VEGFR	Pazopanib	II	Pazopanib as second line therapy in patients with metastatic prostate cancer	NCT00945477
c-MET	Cabozantinib	II	Efficacy of cabozantinib in castrate-resistant prostate cancer metastatic to the bone	NCT01428219
PDGFR, VEGFR	Sunitinib	II	To identify if Sunitinib is an important agent in advanced prostate cancer	NCT00299741
Flt3	Tandutinib	II	To see if Tandutinib works in progressive metastatic cancer and bone metastasis	NCT00390468
SRC	KX2-391	II	Safety and efficacy in patients with CRPC	NCT01074138
SRC	Saracatinib	II	Efficacy in metastasis inhibition in CRPC patients	NCT01267266
FAK	PF-00562271	I	Safety, pharmacokinetic, and pharmacodynamic trial of FAK inhibitor in advanced prostate cancer	NCT00666926

Saracatinib

Saracatinib (AZD0530) is a well-tolerated and potent oral SFK inhibitor that has been tested in Phase II clinical trials for several cancers. Saracatinib is a highly selective and reversible ATP competitive inhibitor of several SFK kinases, including SRC, YES, and LCK. Saracatinib has demonstrated potent inhibitory effects on cell motility, invasion, and metastasis in preclinical models of prostate cancer [108, 109].

A phase II trial of Saracatinib in 28 patients with advanced castration-resistant prostate cancer was conducted [110]. 32 % of patients on this trial had prior docetaxel-based chemotherapy. Median progression-free survival time was 8 weeks. Five patients had transient PSA reduction <30 %. Therefore, Saracatinib has insufficient activity as a single agent in treatment of patients with advanced prostate cancer. Further development in prostate cancer would require rational drug combinations or identification of patient populations who may benefit from SRC inhibition.

KX2-391

KX2-391 (KX-01) is a novel peptidomimetic compound that inhibits SRC kinase signaling and microtubule polymerization. It has a broad range of activity in cancer cell lines, including those that are resistant to commonly used chemotherapeutic agents. Preclinical studies have highlighted the ability of KX2-391 to inhibit the proliferation and metastasis of prostate cancer cell lines in mouse xenograft models. A Phase II clinical trial is underway to evaluate the safety and efficacy of KX2-391 in patients with bone-metastatic CRPC who have not had prior chemotherapy.

Bosutinib

Bosutinib (SKI-606, Bosulif) is a selective dual SRC/ABL inhibitor. It is approved by the FDA for treatment of Philadelphia chromosome-positive chronic myelogenous leukemia (CML). The efficacy of Bosutinib for inhibition of prostate cancer cell growth and bone metastasis was examined in preclinical models [111]. Bosutinib decreased SRC activation, cell proliferation, migration, and invasion of prostate cancer cells. It also significantly decreased phosphorylation of multiple signaling proteins that are important for prostate cancer including AKT, MAPK, and FAK and inhibited expression of tumor progression-associated genes uPAR, MMP-2, MMP-9, N-cadherin, IL8, and TGF-beta in prostate cancer cells. Bosutinib is currently in clinical trials for breast cancer but has yet to be tested in trials for prostate cancer.

TEC Family Kinase Inhibitors

Dasatinib

Although Dasatinib was originally designed to target BCR-ABL and SRC kinases, a later study showed that, besides ABL and SRC kinases, BTK and TEC are two major targets of Dasatinib in CML cells [105]. BMX/ETK, another member of TEC family kinases expressed in prostate cancer cells, was not identified in this screen, possibly due to its low expression in CML cells. Threonine 489 in ETK (corresponding to T474 in BTK and T442 in TEC) is critical for Dasatinib susceptibility

and thus may be likely inhibited by Dasatinib in prostate cancer cells. It is possible that better efficacy of Dasatinib in treatment of various cancers may be due to its inhibition spectrum, which is broader than other SFK inhibitors.

Ibrutinib

Ibrutinib (PCI-32765) is an orally bioavailable small-molecule irreversible inhibitor of BTK with potential antineoplastic activity. It binds to BTK, forms a covalent bond with Cysteine 481 in the kinase domain of BTK, and inhibits BTK activity. Preliminary clinical trial data showed that Ibrutinib was effective in treatment of several types of B cell lymphoma. Currently, a Phase III trial is underway to evaluate the efficacy of Ibrutinib as a monotherapy for patients with relapsed CLL or SLL. The effects of Ibrutinib on other members of TEC family kinases expressed in prostate cancer cells have yet to be tested. The TEC family kinases are highly homologous in the kinase domain. The Cysteine 481 of BTK covalently targeted by Ibrutinib is conserved among these kinases; it is likely that Ibrutinib may also inhibit the activity of other members of this family such as ETK/BMX. There is a compensatory upregulation of ETK and thus tumor growth in prostate cancer cells after castration and knockdown of ETK expression via shRNAs attenuates this growth. If Ibrutinib could target ETK in prostate cancer cells, it may potentially be used as a combined therapeutic agent to block the activation of a compensatory pathway during androgen ablation therapy.

FAK Family Kinase Inhibitors

PF-00562271

PF-00562271 (PF-562271) exhibits the selective inhibitory effects on FAK and PYK2 tyrosine kinase activity with IC50s of 1.5 and 14 nM, respectively. In preclinical models of prostate cancer, PF-00562271 inhibits FAK phosphorylation, tumor growth, and bone metastasis in a dose-dependent manner. However, it failed to inhibit cancer cell growth in tissue culture or induce apoptosis in adherent cells when used at concentrations that effectively inhibited FAK activity. However, tumor cell growth was blocked by PF-00562271 under conditions of anchorage-independent growth [112]. The pharmacokinetics, safety, and pharmacodynamics of PF-00562271 were evaluated in a cohort of patients with advanced solid cancers, including prostate cancer. PF-00562271 was found to be well tolerated along with food. Further studies would delineate its efficacy in prostate cancer [113].

VEGF and VEGFR Inhibitors

Bevacizumab and Aflibercept

Bevacizumab (Avastin) and Aflibercept (Zaltrap, VEGF-trap, Eylea) are antibody-based antagonists that bind to VEGF-A and VEGF-B thereby preventing the binding of these ligands to their receptors and inhibiting angiogenesis. Elevated VEGF in plasma and urine is an independent indicator of poor prognosis for patients with CRPC [114]. Despite potent anti-angiogenic and antitumor activity by both antibodies in various preclinical models, Bevacizumab did not show survival advantage in chemotherapy-untreated CRPC in a Phase III clinical trial [115]. Aflibercept also failed to extend overall survival in combination with docetaxel and prednisone in a randomized Phase III trial.

Sunitinib

Sunitinib (SU11248, Sutent) is an oral multitargeted inhibitor of VEGFR, PDGFR, and several other kinases. It is approved for treatment of advanced renal cell carcinoma and imatinib-resistant or imatinib-intolerant gastrointestinal stromal tumor. Sunitinib alone or in combination with docetaxel inhibits growth of CRPC xenografts [116]. Two Phase II studies in predominantly metastatic castration-resistant patients showed single agent promising activity of sunitinib as a monotherapy for the patients with metastatic CRPC progressing after prior docetaxel treatment [117, 118]. Another Phase I/II trial of sunitinib in combination with docetaxel demonstrated encouraging efficacy in treatment of patients with chemotherapy-naive metastatic CRPC with promising response rate and survival benefit [119]. However, in a subsequent Phase III trial on treatment patients with progressive mCRPC after docetaxel-based chemotherapy, sunitinib in combination with prednisone did not improve overall survival, despite an improvement in median progression-free survival, when compared with prednisone alone. Further evaluation will be needed to determine whether selected subpopulations may benefit from this treatment.

MET Family Kinase Inhibitors

Cabozantinib

Cabozantinib (XL184, Cometriq) is an orally bioavailable small molecule inhibitor for c-MET and VEGFR2. It has shown potent anti-angiogenic, anti-invasive, and anti-proliferative activity in various preclinical models [120]. It was approved by

the FDA in 2012 for treatment of progressive, metastatic medullary thyroid cancer. A number of clinical trials are ongoing to evaluate the effect of cabozantinib either as single therapy or in combination with standard therapy in different stages of prostate cancer including bone metastasized prostate cancer. Cabozantinib demonstrated dramatic and rapid effects on bone scan lesions in a high proportion of patients and significantly improved progression-free survival in a Phase II trial [121]. A significant reduction in risk for bone-related pain and other improvements in quality of life among the men treated with cabozantinib were reported. Cabozantinib is expected to move to Phase III trials in the near future.

Crizotinib

Crizotinib (PF-2341066, Xalkori) is an oral selective, ATP-competitive small molecule dual inhibitor of ALK and MET family kinases. Crizotinib is approved by the FDA for the treatment of ALK-positive non-small cell lung cancer. PF-2341066 inhibits proliferation of human prostate cancer cells and phosphorylation of c-MET in these cells in a dose-dependent fashion. The effect on cell proliferation was more pronounced in androgen insensitive cells. PF-2341066 also significantly inhibited tumor growth in preclinical animal models of prostate cancer, with more efficacy in castrated animals [122]. It warrants further investigation whether PF-2341066 has anti-proliferative efficacy in combination with androgen ablation therapy for advanced prostate cancer.

BMS-777607

BMS-777607 is a selective ATP-competitive inhibitor for MET family kinases and ALX family kinases. It blocks the autophosphorylation of c-MET and demonstrates selective inhibition of proliferation in Met-driven tumor cell lines as well as invasion of various cancer cell lines including prostate cancer [123]. A Phase I/II clinical trial is undergoing to determine the maximum tolerated dose and activity of BMS-77607 in patients with advanced or metastatic solid tumors including CRPC.

EGFR Family Kinase Inhibitors

Lapatinib

Lapatinib (Tykerb) is a small molecule dual kinase inhibitor of EGFR and HER2 and is used in a combination therapy for HER2-positive breast cancer. Efficacy of Lapatinib was evaluated in chemotherapy naïve CRPC patients in a Phase II study

[124]. One of 21 patients showed a PSA decline >50 % and another patient showed a 47 % decrease in PSA. Further clinical trials would help in understanding the patient subset most responsive to lapatinib therapy and potential combination treatment options with standard of care treatment.

Trastuzumab

Trastuzumab (Herceptin) is a humanized monoclonal antibody to the HER2 receptor and promotes internalization of HER2. It is approved for the treatment of HER2-positive breast cancer. Several Phase II clinical trials have been conducted to test whether Trastuzumab has efficacy on HER2-positive prostate cancer [125–127]. The results from these trials are discouraging. Trastuzumab appears to be not effective as a single agent or in combination with taxene for the treatment of patients with CRPC. One of challenging issues in these trials is how to reliably identify the patients with high HER2 expression in metastatic lesions. A study on analyzing HER2 expression in 126 primary prostate and breast cancer tissues showed that the expression level was comparable in all prostate tumor types and corresponded to the level of expression in breast tumors without HER2 amplification [128]. The frequency of HER2 amplification is extremely low in prostate cancer and only one case in this cohort. An average 20-fold increase of HER2 mRNA levels was detected in breast tumors with HER2 amplification when compared to prostate or breast tumors without HER2 amplification. Therefore, the expression of HER2 protein in prostate cancer is relatively low due to low frequency of amplification in the primary tumors. Further development of trastuzumab for the treatment of patients with metastatic prostate cancer is not feasible until a more reliable and practical approach is established to stratify patients with HER2-overexpressing metastatic tumors [125, 129].

Pertuzumab

Pertuzumab (2C4, Perjeta) is a monoclonal antibody that targets the extracellular dimerization domain (subdomain II) of HER2 and thereby blocks heterodimerization of HER2 with other EGFR family members. Pertuzumab is approved for use in combination with trastuzumab and docetaxel to treat patients with HER2-positive metastatic breast cancer. In a Phase II clinical trial, the efficacy and safety of pertuzumab as a single-agent in treatment of CRPC patients who failed in prior chemotherapy was assessed [130]. Pertuzumab was well tolerated with no disease progression in several patients for more than 23 weeks. Retrospective analysis suggested a prolonged median survival time with pertuzumab compared to historical controls. Therefore, inhibition of HER dimerization may be a promising strategy for treatment of patients with CRPC.

AZD8931

AZD8931 is a reversible ATP-competitive inhibitor of EGFR, ErbB2, and ErbB3. AZD8931 can simultaneously and equipotently inhibit signaling events mediated by these three ErbB kinases and exert broad antitumor activity in vitro and in vivo preclinical models. AZD8931 is significantly more potent than gefitinib or lapatinib in growth inhibition in various cancer cell lines and xenograft models [131]. AZD8931 provides the opportunity to investigate whether simultaneous inhibition of ErbB receptor signaling could be of clinical utility, particularly in the majority of solid tumors without ErbB2 amplification. A Phase I clinical trial is undergoing to assess safety, tolerability, and pharmacokinetics of AZD8931 in patients with advanced solid tumors.

Closing Remarks

Tremendous progress has been made in detection, characterization, and inhibition of tyrosine kinases that are deregulated in prostate cancer in the past two decades. Despite some promising results from various clinical trials, targeted therapy against tyrosine kinases in prostate cancer is in the infancy stage. We still have a long way to go before we can translate our knowledge of these kinases into effective therapy for prostate cancer. Although the methods for detecting tyrosine kinase expression at various molecular levels are well established, it is still quite challenging to faithfully monitor tyrosine kinase activity temporally and spatially during disease progression or in response to therapy. Delineation of signaling pathways controlled by individual kinases has laid the foundation for understanding the effects of perturbation of a given kinase on the global signaling network. Efficacy of therapeutic intervention of tyrosine kinases-mediated signaling pathways or network is heavily dependent on the addiction level of the targeted kinases in prostate cancer cells. Therefore, establishment of reliable and practical means to stratify patient population based on molecular signatures present in their specimens is critical for improving efficacy of targeted therapy. Furthermore, development of new therapeutics targeting multiple key kinases to prevent the activation of the compensatory machinery in cancer cells may hold promise for effective treatment of prostate cancer.

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Chapter 12
Human Prostatic Acid Phosphatase in Prostate Carcinogenesis

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Abstract Human prostatic acid phosphatase (PAcP) is classically known as a prostate epithelium-specific differentiation antigen and was used as a surrogate marker for detecting prostate cancer (PCa) and monitoring its progression until the availability of prostate-specific antigen. Mature human PAcP protein is a 100 kDa glycoprotein containing two subunits of approximately 50 kDa each. Classically, two forms of human PAcP proteins have been identified: the cellular form (cPAcP) and the secretory form (sPAcP). Recent studies reveal the existence of a transmembrane form (TM-PAcP). While the function of sPAcP and TM-PAcP in human remains under further investigation, cPAcP functions as a neutral protein tyrosine phosphatase in PCa cells and dephosphorylates human epidermal growth factor receptor-2 (HER-2/ErbB-2/Neu) resulting in decreased cell growth as well as tumor suppression. Clinically, cPAcP levels decrease in PCa tissues and correlate with PCa progression, despite elevated levels of sPAcP in circulation. Data from xenograft animal models validate the tumor suppressor activity of cPAcP in prostate carcinomas. Further, activation of ErbB-2 upon knockdown of cPAcP expression results in a castration-resistant phenotype. Expression of PAcP is regulated by different factors in human PCa cells. PAcP is also a useful immunogen in PCa immunotherapy. Further investigation of the regulatory mechanism of cPAcP expression will likely provide valuable insights into novel PCa therapy.

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Abbreviations

Ab	Antibody
AcP	Acid phosphatase
ADT	Androgen deprivation therapy
APCs	Antigen-presenting cells
CRPCa	Castration-resistant prostate cancer
DHT	5α-dihydrotestosterone
EGF	Epidermal growth factor
EGFR	EGF receptor
FBS	Fetal bovine serum
HDAC	Histone deacetylase
HER-2/ErbB-2/neu	Human epidermal growth factor receptor-2
PAcP	Prostatic acid phosphatase
PCa	Prostate cancer
PI3K	Phosphoinositide 3-kinase
pIs	Isoelectric point
PKC	Protein kinase C
PSA	Prostate-specific antigen
PTP	Protein tyrosine phosphatase
p-Tyr	Phosphotyrosine
TM-PAcP	Transmembrane PAcP
Tyr-P	Tyrosine phosphorylation

Introduction

Prostatic acid phosphatase (PAcP; E.C.3.1.3.2) is known classically as a prostate-specific differentiation antigen in differentiated prostate epithelia [1, 2]. Human PAcP protein is synthesized in differentiated columnar epithelia of the prostate gland [2–6]; some of which is secreted into prostatic fluid as the secretory form (sPAcP) and the rest is retained intracellularly as the cellular form (cPAcP) [7]. Recent studies have revealed a transmembrane form of PAcP (TM-PAcP) [8]. Since PAcP biochemically hydrolyzes a broad variety of small organic phosphomonoesters under acidic conditions, this enzyme is known as an AcP [3, 9–11].

PAcP levels are very low in normal circulation. In 1936, Gutman and colleagues made the seminal observation that human PAcP activity in serum is significantly increased in PCa patients, especially those with bone metastases [12]. Subsequently, Huggins and colleagues reported that the circulating PAcP activity correlates with prostate tumor progression [13]. Since then, serum PAcP has been studied extensively as a surrogate marker for the diagnosis of PCa until the availability of PSA [14].

PAcP expression is positively associated with prostate epithelial cell differentiation. Prior to puberty, cPAcP is expressed at a low level. In normal, well-differentiated human prostate epithelial cells, the high level of cPAcP protein is in agreement with slow cellular growth [1, 2, 15]. In prostate adenocarcinoma cells cPAcP expression is lower than in adjacent noncancerous cells, despite elevated sPAcP activity in circulation [6, 16–18]. Studies have suggested that cPAcP acts as a tumor suppressor in PCa cells [19–21]. Several lines of evidence collectively support the concept that cPAcP functions as a histidine-dependent PTP in prostate epithelia and regulates its tumorigenicity by dephosphorylating p-Tyr of human ErbB-2 (also known as HER-2 or neu protein) at physiological pH [19, 21–24]. cPAcP also plays a critical role in regulating the cross-talk between androgens and Tyr-P signaling. In parallel, numerous studies have shown a therapeutic potential of PAcP for the treatment of PCa [19, 21, 25–28]. Hence, in this chapter, we review the structure and regulation of PAcP isoforms in prostate epithelia and the function and therapeutic role of cPAcP in human PCa.

Human Prostatic Acid Phosphatase

Physiology of PAcP

AcPs are a group of enzymes that biochemically hydrolyze phosphomonoesters optimally at acidic pH. In human cells, AcPs can be divided into at least five isoforms, including erythrocytic, lysosomal, prostatic, macrophagic, and testicular AcPs [2, 29, 30]. The mature form of PAcP is a glycoprotein consisting of two subunits of approximately 50 kDa each and is synthesized in differentiated columnar prostate epithelia [3, 5, 17, 31]. There are two forms of PAcP protein in well-differentiated human prostate epithelia: the cellular form (cPAcP) and the secretory form (sPAcP). Recent results reveal the possible existence of a transmembrane form (TM-PAcP) [8].

The physiological level of PAcP is negligible in fetal tissue and young males. After puberty, cPAcP level can reach approximately 0.5 mg/g of wet tissue in normal, well-differentiated prostate epithelia [2, 15]. sPAcP is predominantly secreted into seminal fluid at approximately 1 mg/ml and has been used as a marker in forensic medicine [7, 14]. In PCa patients, the circulating level of sPAcP is elevated and correlates with the stage of PCa. Hence, PAcP has received much attention and has served as a surrogate marker for PCa [12–14].

Expression and Distribution of PAcP in Human Tissues

While PAcP is considered as a prostate-specific differentiation antigen, studies of PAcP expression in non-prostate tissues have yielded inconsistent results. Solin et al. [32] showed by RNA blot analysis that there is no detectable hPAcP mRNA in

human liver, lung, pancreatic cancer tissue, placenta, breast cancer cells, mononuclear blood cells, or acute promyelocytic leukemia cells. Similarly, Zelivianski et al. [33] could not detect hPAcP mRNA expression in spleen, thymus, testis, ovary, small intestine, colon, or peripheral blood leukocyte by northern blotting.

On the contrary, immunologic studies demonstrate the positive reactivity of hPAcP Ab in some non-prostate cells such as leukocytes, kidney, spleen, placenta, pancreas, liver, stomach, granulocytes, neutrophils [34–38], male anal gland and in urethral gland of both sex [39], crypt epithelium of the duodenum [40], pancreatic islet cell carcinomas [41], and breast tumor cells [34]. Nevertheless, it has been proposed that the reactivity of hPAcP Ab in non-prostatic cells does not recognize the authentic hPAcP, but an immunologically cross-reactive AcP [22, 32, 42, 43], e.g., lysosomal acid phosphatase, a transmembrane phosphatase expressed in almost all tissues and cell types [38]. Supportively, purified AcPs from human spleen and lung having a similar molecular weight as hPAcP share at least one common antigenic epitope with hPAcP [22].

Recently, Graddis et al. [44] using quantitative RT-PCR reported that hPAcP is expressed at moderate to high levels in both normal and malignant prostate tissues. In non-prostate normal tissues examined, bladder cells express the highest ratio relative to prostate, though the expression level is still 50-fold lower than prostate [44]. The ratio of normal prostate PAcP mRNA to normal kidney PAcP mRNA is 178, which is comparable to the ratio of 192 reported previously [45]. The hierarchical tissue distribution of hPAcP mRNA by PCR in normal tissues is prostate >>>bladder>kidney>pancreas>cervix = testis>lung = ovary [44], which is similar to previous analyses prostate>>>placenta>kidney>testis>pancreas>small intestine = leukocytes>lung>ovary [45]. Among the tumor samples analyzed, cervical tumors express PAcP mRNA at the level similar to that seen in normal bladder; i.e., the level is less than 2 % of that in normal prostate [44]. Due to the clinical importance of hPAcP in PCa therapy and other medical applications, further experiments should clarify the identities of these proteins by determining their sequence.

Expression of PAcP in Prostate Epithelia

Immunohistochemistry staining has demonstrated that hPAcP is primarily localized in the differentiated columnar epithelial cells of prostate [37, 43, 46–48]. In situ hybridization analyses confirmed that hPAcP mRNA is detected in the glandular, ductal epithelial cells of prostate, and that the stromal cells are devoid of this mRNA [6]. An electron microscopic study showed that hPAcP is in the microvilli lining and vesicular bodies of apical cells in normal prostate [46]. The existence of human cPAcP in the cytosolic fraction has been clearly demonstrated by various biochemical approaches including sub-fractionation [9, 21]. Due to the importance of cPAcP in regulating Tyr-P signaling in PCa cells, it is imperative to clarify the subcellular localization of cPAcP where it interacts with ErbB-2 for growth regulation.

Structure of Human Prostatic Acid Phosphatase

Biochemical Characterization of Human PAcP Gene and mRNA

The human PAcP gene is located at chromosome 3q21–q23 [49] and has a size of more than 40 kb and distributed over 10 exons [50, 51]. Exon 1 encodes for the signal peptide and the first eight amino acids of the protein. Exons 2–10 encode the rest of the coding regions and 3′-untranslated region (Fig. 12.1). Several PAcP cDNA clones have been obtained in which additions, deletions, and/or substitutions of nucleotides [49, 52–54] lead to the heterogeneity of amino acid residues. Interestingly, two different signal peptide sequences have been identified [52–54]. The biological significance of these heterogeneities in PAcP sequence requires further investigations.

In human LNCaP prostate carcinoma cells, the major transcription start site is located at 50 nucleotides upstream of the gene's ATG codon [55]. In normal differentiated human prostate epithelia, two species of PAcP mRNA are detected by Northern blot analysis with molecular sizes of 2.4 kb and 3.3 kb, essentially due to the variation in the number of Alu repeats in the 3′-noncoding sequence [32]. In prostate carcinomas, only expression of the 3.3 kb species is detected, which is lower than in noncancerous cells [32, 33, 52, 56]. The biological significance and the molecular mechanism of the loss of 2.4 kb PAcP mRNA expression in PCa cells is not clear.

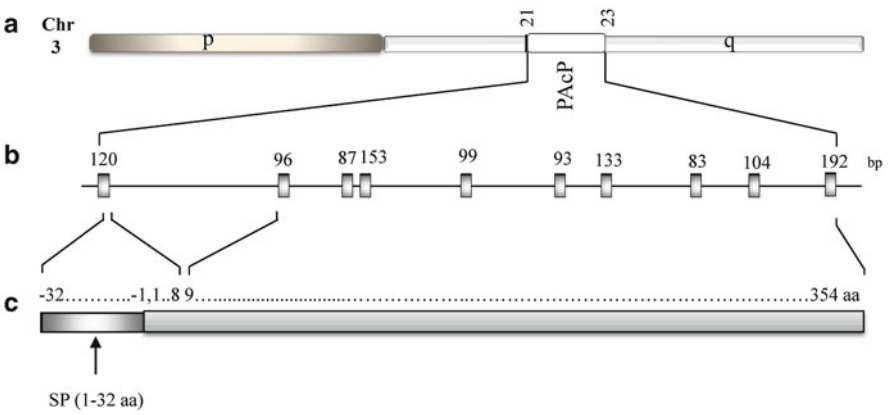

Fig. 12.1 Schematic diagram of human prostatic acid phosphatase (PAcP) gene and protein. (**a**) Localization of the hPAcP gene in the q-arm of the chromosome 3 (Chr 3). (**b**) hPAcP gene encoded by 10 exons. The number of nucleotides in each exon was noted. (**c**) The full length of hPAcP protein consisting of 386 amino acids with 32 amino acid signal peptides (SP). The signal peptide and the first eight amino acids were encoded by exon 1 and the rest of amino acids were coded by exons 2–10

Structure of the PAcP Protein

The PAcP protein is initially translated as a precursor form with 386 amino acids containing a 32-amino acid signal peptide, and the mature PAcP protein of 354 amino acids has a calculated molecular mass of 41,126 Da (Fig. 12.1). The signal peptide directs the nascent PAcP polypeptide into rough endoplasmic reticulum, which provides an environment for posttranslational modifications of PAcP protein [52, 57]. These modifications provide stability for the PAcP protein, where deletion of the signal peptide sequence from cDNA results in an extreme low level or nondetectable PAcP protein [58, 59], (Lingappa, Vishwanath and Lin, Ming-Fong, Unpublished observations). It has been proposed that the signal peptide directs secretion of PAcP protein [59]; moreover, no cPAcP protein is found intracellularly [58, 59]. These data support the concept that the function of signal peptide is primarily responsible for directing the nascent peptide via rough endoplasmic reticulum for its various post-translational modifications, including glycosylation, which stabilizes PAcP protein [58].

Sequence analysis has revealed that hPAcP protein contains three asparagine-linked glycosylation sites (62Asn–Glu–Ser64, 188Asn–Phe–Thr190, and 301Asn–Glu–Thr303) and 6 cysteine residues forming two disulfide bonds (Cys129–340 and Cys314–319) and two free residues (Cys183 and Cys281). The glycosylation and disulfide linkages support the structural conformation and the stability of PAcP protein. Molecular sieving under native and denaturing conditions indicates that hPAcP is a dimer consisting of two subunits of similar molecular size [3, 9, 11, 60]. Analyses of the crystal structure of hPAcP reveal that each subunit has two domains: the larger domain is α/β type composed of a central seven-stranded mixed β-sheet with helices on both sides; while, the second, smaller one contains six α-helices and is formed mostly by long-chain excursions (residues 125–227) from the first domain and α-loop between residues 16–38 with no secondary structural elements [61].

PAcP Isoforms: Cellular, Secretory, and Transmembrane Forms

Recent studies have revealed that the signal peptide of hPAcP protein can direct differential biosynthetic pathways, which results in different biological functions of PAcP protein, depending on the growth environments [56], (Lingappa, Vishwanath and Lin, Ming-Fong, Unpublished observations). cPAcP and sPAcP proteins exhibit unique antigenic epitope(s), yet they share partial cross-reactivities [9]. Biochemically, they exhibit different, while overlapping p*I*s [9, 62].

The level of PAcP in normal circulation is negligible, while it is elevated in PCa patients and correlates with clinical progression [12, 13, 63, 64]. Thus, circulating PAcP has served as a surrogate marker for PCa detection prior to the

availability of PSA [14, 65, 66]. The tumor-associated PAcP exhibits different biochemical properties from normal species and is hyper-glycosylated, including sialylation [11]. This hyper-sialylation prolongs the half-life of sPAcP protein in circulation, which contributes to the elevated PAcP level in the circulation of PCa patients despite its decreased mRNA level [11, 67]. sPAcP and cPAcP exhibit different sensitivities to endoglycosidase [Garcia-Arenas, Renee and Lin, Ming-Fong, Unpublished observations]. The biological significance of glycosylation involved in PAcP function and subcellular localization deserves further studies.

Large amounts of PAcP protein are found in normal prostate tissues by immunohistochemistry staining and biochemical analysis of tissue homogenates [9, 62, 68, 69]. The presence of cytosolic cPAcP protein is further validated by sub-fractionation approaches, including ultracentrifugation [21]. Additionally, immunocytochemistry staining of intact, nonpermeabilized LNCaP PCa cells shows no significant staining of hPAcP. On the contrary, intensive staining is seen in the cytosolic area of permeabilized cells with higher intensity of staining in higher density cells [21, 70, 71]. Therefore, cPAcP is localized intracellularly and has served as a useful marker for identifying the prostate origin of metastatic cancer [1, 2, 14]. In prostate carcinomas, intracellular PAcP protein level decreases, correlating with PCa progression [6, 16]. The decreased protein level is at least in part by the decrease of mRNA [32]. Because of the importance of cPAcP in tumorigenicity and androgen sensitivity of PCa cells, the molecular structure of cPAcP relating to sPAcP should be further investigated.

Quintero et al. [8] reported the existence of a PAcP-spliced variant, which is a type I transmembrane protein in many mouse tissues. However, its expression profile in human tissues other than prostate is not yet known. Immunostaining with an anti-PAcP Ab showed PAcP expression in human skeletal muscle cells. They further demonstrated that PAcP colocalizes with lysosomal associated membrane protein 2. None-the-less, expression of PAcP is extremely low in skeletal muscle cells, and importantly, exhibits no lysosomal localization [1, 2, 38]. These observations have raised a concern that the staining may be due to a partial cross-reactivity of polyclonal Ab [22]. It has been further proposed that the active site of TM-PAcP is localized and functioning extracellularly [20]. Biochemical analyses have demonstrated that no phosphatase activity is significantly detected when small organophosphate substrates are incubated with intact LNCaP cells. These results collectively suggest that in prostate epithelia only a very low amount, if any, of the active domain of hPAcP faces extracellularly. While the decreased expression of classical PAcP mRNA and protein correlates with PCa progression, the expression of TM-PAcP mRNA is not significantly changed, indicating that TM-PAcP is not involved in prostate carcinogenesis [8]. A functional characterization of TM-PAcP in prostate epithelia utilizing its specific monoclonal Ab is required for further investigation.

Biological Function of Prostatic Acid Phosphatase Isozymes

Cellular PAcP (cPAcP) as a Growth Regulator and Tumor Suppressor in Prostate Cancer Cells

Expression of PAcP is correlated with the differentiation of normal prostate cells. In PCa, cPAcP levels of both mRNA and protein are decreased, compared to nonmalignant cells [6, 16, 63, 69, 72–74]. It has been proposed that normal prostate epithelia, having a low level of cPAcP, are at high risk of carcinogenesis [72]. In human PCa cell lines, cPAcP levels are correlating inversely with cell growth rates [16, 19, 21, 56]. Results of increased PAcP expression by cDNA transfection and decreased cPAcP expression by antisense cDNA or shRNA knockdown in PCa cells validate the growth regulatory role of cPAcP in PCa cells [17, 21, 71].

Several studies of clinical archival specimens showed that decreased cPAcP expression correlates with increased tumorigenicity and cancer progression [2, 6, 16, 17, 75]. Conversely, expression of cPAcP correlates with decreased tumorigenicity of PCa cells in xenograft animals [16]. Igawa et al. [19] further explored the direct tumor suppression activity of cPAcP in a xenograft animal model. Importantly, in a PAcP-knockout mouse model, the prostate develops adenocarcinomas [20]. The data collectively demonstrate that cPAcP expression suppresses the growth and tumorigenicity of PCa cells. This provides an explanation for the clinical phenomenon that the expression levels of cPAcP inversely correlate with the stage of PCa as well as its advanced progression under androgen deprivation therapy (ADT) [6, 72].

Further studies have revealed that cPAcP is involved in regulating androgen-stimulated proliferation of human PCa cells [76–78]. Expression of cPAcP by cDNA transfection in androgen receptor (AR)-positive, androgen-independent LNCaP C-81 cells results in restoring the androgen sensitivity, i.e., the cell growth is sensitive to androgen treatment [16, 78]. Conversely, an androgen-independent phenotype is obtained by knockdown of cPAcP expression by shRNA in androgen-senstitive LNCaP C-33 cells [21]. Thus, androgen-induced proliferation of prostate epithelia is at least in part due to an androgen effect on decreasing cPAcP activity [16, 71, 78]. The data taken together support the concept that cPAcP plays a critical role in regulating the basal as well as the androgen-stimulated proliferation of human PCa cells.

Transmembrane PAcP (TM-PAcP) as an Analgesic in Mice

While TM-PAcP is detected in several tissues from mice, thus far it has been detected only in prostates of humans [8]. Interestingly, PAcP knock-out ($PAcP^{-/-}$)

mice display enhanced noxious thermal sensitivity and sensitization through inactivation of adenosine A1 receptor (A1R) and phospholipase C-mediated elevation of phosphatidylinositol 4,5-bisphosphate (PIP2) [20, 79, 80]. It has been proposed that TM-PAcP is the fluoride-resistant acid phosphatase and functions as phosphoadenosine phosphatase upon expression in human embryonic kidney HEK293 cells [20]. Considering the long-lasting antinocieptive effect of secretory PAcP in the naïve mouse model, it has been proposed that recombinant PAcP can be used to treat chronic pain [20, 81, 82]. However, it is not known if TM-PAcP is indeed expressed in the corresponding human tissues and cells. The expression profile of TM-PAcP in human tissues and cells should be analyzed.

Role of Secretory PAcP (sPAcP) in Sperm Motility, HIV Transmission, and Forensic Marker

Due to the large amount of sPAcP protein in seminal fluid [83], it has been suggested that PAcP plays a physiological role in fertility [84] and may affect the motility of sperm [20, 85]. Dave and Rindani [86] observed that phosphatase activity is maximal in azoospermic men, and that this activity is decreased as the sperm number (concentration) increases. However, there is no significant difference in PAcP activity in seminal plasma between normal and vasectomized patients [87].

Importantly, a proteolytically cleaved PAcP peptide, PAPf39, which forms amyloid fibrils called Semen-derived Enhancer of Viral Infection (SEVI), can enhance the HIV's ability to infect human cells by five orders of magnitude [88]. This observation is in agreement with a previous report that HIV replication component is detected at a tenfold higher concentration in seminal fluid than in blood, even in the presence of an antiretroviral drug [89]. Alternatively, PAcP may increase the pH of the vagina [90]. Thus, this postintercourse neutralization of pH may allow a female-to-male transmission of HIV [91]. The role of sPAcP in sexually transmitted diseases requires further investigation.

It should be noted that secretory PAcP can also serve as a forensic marker. Due to the large quantity of sPAcP in seminal fluid, and also due to its specificity of expression in males, secretory PAcP was investigated and served as a surrogate marker in forensic medicine for sexual assault [92]. This was supported by the observations that elevated levels of AcP activity persist in the vaginal pool after sexual intercourse and in semen stains [93–100]. Nevertheless, there are some concerns, for example, the potential cross reactivity with other acid phosphatases and the instability of its enzymatic activity. The subsequent advancements including the enhancement in enzymatic assays and the development of detection methods such as ELISA, counter-immunoelectrophoresis, and radioimmunoassays made PAcP as a useful surrogate marker in forensic medicine [101–104]. Nevertheless, the discovery of PSA (also called γ-seminoprotein; γ-SM) in seminal fluid, due to its

long-term stability (identifiable for as long as 1 year) and easy identification, replaced PAcP as the forensic marker [14, 105–108].

Biochemical Characterization of Human Prostatic Acid Phosphatase Protein

PAcP: A Histidine-Dependent Neutral Protein Tyrosine Phosphatase

PAcP, which belongs to the histidine phosphatase superfamily, uses an active-site histidine in catalyzing the transfer of a phosphoryl group from phosphomonoesters to water at acidic pH. The copurification of cPAcP protein with the majority of PTP activity from noncancerous prostate tissue and purified PAcP protein from seminal fluid and tissue exhibiting the endogenous PTP activity together indicate that PAcP is an authentic PTP [109, 110]. Biochemical characterizations show that PAcP dephosphorylates p-Tyr of EGFR with a neutral pH optimum, supporting the concept that cPAcP can indeed function as a neutral PTP in cells [111]. Further, in PAcP K/O mice, Tyr-P activity is increased in prostate cells, suggesting that intracellular cPAcP functions as PTP [20, 112]. Several lines of evidence together support the notion that cPAcP indeed functions as a neutral PTP in prostate epithelia [17, 110, 113].

Structural analyses of PAcP protein reveal that it contains neither the PTP signature motif, $C(X)_5R(S/T)$, nor the extended active site signature sequence for the dual-specificity phosphatases, $VXVHCXXGXXRS(X)_5AY(L/I)M$ [52, 57, 114, 115]. Chemical titration experiments revealed that PAcP has two reactive sulfhydryl groups [116]. It was hypothesized that Cys183 is essential for the PTP activity of PAcP. Nevertheless, studies by site-directed mutagenesis determined that neither Cys183 nor Cys281 plays a role in the phosphatase enzymatic activity [58].

Covalent modifications and phosphoenzyme trapping studies revealed that PAcP contains histidine and carboxylic acid residues in the active site [117–119]. The role of His12 in both AcP and PTP activities is clearly evidenced by site-directed mutagenesis [58]. The His12 imidazole ring provides a pair of electrons for nucleophilic attack to the phosphate group. Cooperatively, Asp258 donates a proton from its carboxyl group to the substrate resulting in the formation of the phosphoenzyme intermediate and the liberation of dephosphorylated substrate. Additionally, Asp258 might also stabilize the phospho-His12 intermediate. Subsequently, the nucleophilic attack of the phosphoenzyme intermediate occurs through a water molecule to release the phosphate group and to return a proton to the Asp258 carboxyl group [116, 120, 121]. The data collectively from chemical modification, site-directed mutagenesis, and X-ray crystallographic approaches suggest the importance of His12 and Asp258 in both AcP and PTP activity of PAcP protein [58, 61, 116,

120–122]. These results further support the concept that PAcP represents a novel histidine-dependent PTP, which uses the same active site as well as the catalytic mechanism of AcP to execute its PTP activity.

ErbB-2/HER-2/neu: An Intracellular Substrate of cPAcPin Prostate Epithelia

Several lines of evidence validate cPAcP as an authentic PTP [17, 21, 109–111, 113, 121, 123]. In PCa cells, cPAcP activity inversely correlates with the p-Tyr level of a 185 kDa protein [23, 111]. The incorporation of purified PAcP protein into PAcP-null DU145 PCa cells results in decreased Tyr-P of 185 kDa protein [23]. The 185 kDa protein was identified to be the ErbB-2 [24]. The notion of cPAcP dephosphorylating ErbB-2 Tyr-P is further supported by ectopic expression of the wild-type cPAcP but not its phosphatase-inactive mutant in PCa cells [16, 58]. Conversely, small interfering RNA or antisense-mediated PAcP knockdown in LNCaP cells results in increased ErbB-2 Tyr-P and subsequently cell proliferation [17, 21]. Additionally, an intratumoral injection of the wild type PAcP, but not phosphatase-inactive mutant, cDNA expression vector in xenograft tumors results in decreased ErbB-2 Tyr-P as well as tumorigenicity [19].

Transient expression of PAcP in PAcP-null PCa cells is associated with decreased Tyr1221/2 and Tyr1248 phosphorylation at ErbB-2 and reduced Tyr-P of p52Shc and cell growth [21]. Knockdown of endogenous PAcP expression by shRNA is associated with elevated Tyr-P of ErbB-2 at Tyr1221/2 as well as Tyr1248 and activation of downstream signaling, including Akt, STAT-3, and STAT-5 [21]. Importantly, reciprocal co-immunoprecipitation analyses showed an interaction between PAcP and ErbB-2 in the same complex under a nonpermissive growth condition [21]. This interaction by co-immunoprecipitation was decreased upon growth stimulation [124]. Thus, the effect of cPAcP on downregulation of PCa cell growth is at least in part through dephosphorylating the p-Tyr of ErbB-2 protein in those cells [16, 21, 24, 58, 124]. ErbB-2 serves as an in vivo substrate of cPAcP in PCa cells [17, 19, 21, 120, 124] (Fig. 12.2).

The cPAcP dephosphorylation model indicates that dimeric cPAcP dephosphorylates two autophosphorylated residues on an activated receptor simultaneously because the presence of a second phosphorylated tyrosyl residue at the C terminus of ErbB-2 can enhance the binding affinity considerably [120]. Phosphopeptide-binding analyses showed that cPAcP has the most favorable binding energy toward the synthesized peptide DNLpYYWD, corresponding to Tyr1221/2 phosphorylation of ErbB-2, with the possibility of acting on Tyr1248 as the additional site [120]. This is supported by kinetic studies on ErbB-2 activation that phosphorylation of Tyr1221/2 is elevated prior to Tyr1248 activation in PAcP-knockdown PCa cells [21]. Alternatively, due to the close proximity of Tyr1221/2 and Tyr1248, elevated phosphorylation on Tyr1248 in PAcP-knockdown cells may be secondary to the

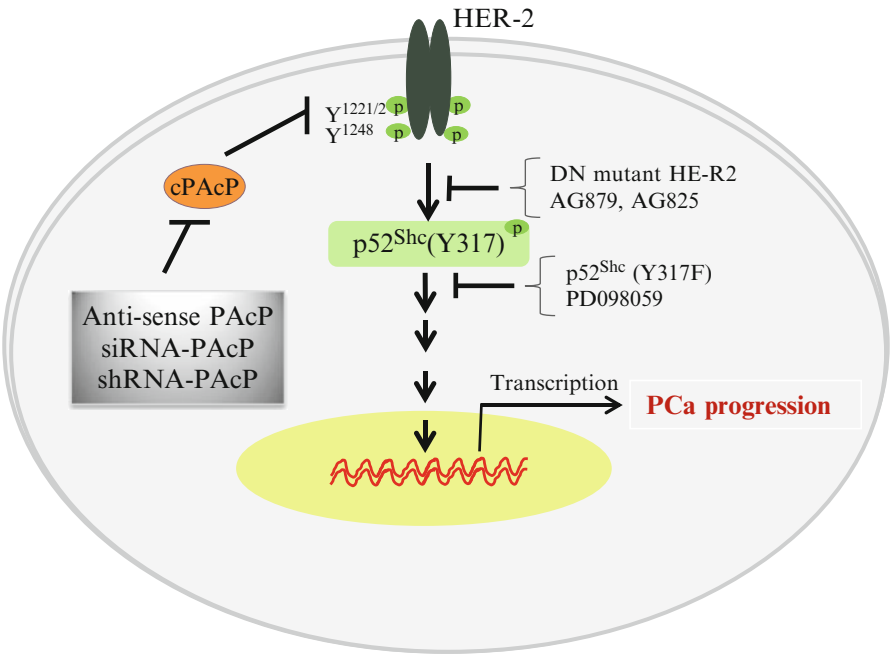

Fig. 12.2 Schematic representation of cPAcP interaction with ErbB-2 in prostate cancer cells. Progression of androgen-sensitive prostate cancer cells towards androgen independence is accompanied by early decrease/loss of cPAcP expression in prostate cancer cells results in hyperphosphorylation of HER-2 on tyrosine residues including Y1221/2 and Y1248 leading to androgen-independent cellular proliferation. Activated HER-2 can transduce its signals via p52Shc (blocked by dominant-negative (DN) HER-2 cDNA transfection or HER-2 inhibitors, AG825, AG879) to activate the downstream pathway (blocked by p52Shc Y317F mutant cDNA transfection or MEK inhibitors)

removal of cPAcP from Tyr1221/2. Further experiments should clarify the molecular mechanism.

ErbB-2 Signaling

Results of several studies clearly support the notion that ErbB-2 plays a critical role in PCa progression despite the fact that ErbB-2 gene is not amplified nor ErbB-2 protein is elevated in most carcinomas. It should be noted that ErbB-2-specific activity is increased as shown by increased overall Tyr-P and downstream signaling in AI PCa cells, higher than that in corresponding AS cells, and this increased Tyr-P is inversely correlated with cPAcP activity [21, 23, 24, 77]. Evidently, knockdown of cPAcP expression by antisense cDNA and siRNA in AS PCa cells leads to increased Tyr-P of ErbB-2, activation of downstream signaling and increased cell growth both in regular medium and in steroid-reduced condition [17, 21]. Thus, increased ErbB-2 protein-specific activity contributes to

advanced CRPCa progression primarily by phosphorylation regulation, including ERK/MAPK, Akt as well as STAT-3 and STAT-5 activation, which leads to advanced PCa cell survival, proliferation, and PSA production under androgen-deprived conditions. In summary, the interaction between cPAcP and ErbB-2 is involved in controlling the basal as well as the androgen-stimulated proliferation of human PCa cells [21, 78]. Aberrant regulation of this interaction can lead to CRPCa progression under ADT.

Regulation of PAcP Expression

The expression of PAcP protein is regulated by multi factors at different levels. Due to the importance of cPAcP protein as a growth regulator in PCa cells, it is vital to delineate its regulatory mechanism for potential clinical applications.

Androgen Regulation of PAcP Expression and Secretion in Prostatic Carcinoma Cells

Since PAcP expression correlates with the differentiation of prostate epithelia after puberty, it has been proposed that the expression and secretion of PAcP is regulated by androgens [2]. The stimulated secretion of PAcP has served as a hallmark of androgen action in prostate epithelial cells for over 6 decades, and the circulating PAcP in PCa patients has been used as a surrogate marker in ADT for about five decades [1, 2, 13].

Lin and Garcia-Arenas [125] made the seminal observation that depending on the cultured cell density, DHT can upregulate or downregulate PAcP mRNA levels in LNCaP cells. These results clarify the inconsistent reports of the opposite regulations of PAcP mRNA by DHT [54, 126]. Nuclear run-on experiments showed that DHT regulation of PAcP expression can occur at the transcriptional level [33]. Further investigation is needed to delineate the molecular mechanism by which cell density modulates androgen regulation of PAcP mRNA level and to examine whether androgens regulate the stability of PAcP mRNA in addition to the transcriptional rate [33].

Sequence analyses revealed that the human PAcP gene promoter DNA within 3 kb upstream of the coding region lacks the canonical TATA box and the GC box, where there are five putative AREs [55, 127]. In prostate carcinoma cells, although the PSA promoter is regulated by androgens [128–130], PAcP expression is not androgen dependent [54, 126, 131]. Utilizing AR-negative, androgen-independent PC-3 and DU145 PCa cell lines, in the absence of androgen receptor or the addition of androgen, the PAcP promoter is highly active as determined by reporter assay. These results demonstrate that in PCa cells, the PAcP gene is regulated in an androgen-independent, responsive manner [70, 126, 132]. Supportively, secretion

of PAcP protein is observed when LNCaP cells are cultured in media supplemented with charcoal-stripped FBS [133] or dialyzed FBS [77, 134] in which steroids and growth factors are significantly reduced. Further, PAcP protein expression is at a high level in cells with serum-free media in the absence of added DHT and even higher than with regular medium containing FBS [54, 77]. These data together support the notion that in LNCaP cells, androgen-stimulated PAcP secretion is via two regulatory pathways: increasing levels of secretory PAcP mRNA and at the same time, promoting the secretory pathway [54].

It has been shown that JFC1 [also designated as synaptotagmin-like protein (slp1)], a Rab27a and PtdIns(3,4,5)P3-binding protein, can modulate androgen-stimulated secretion of sPAcP in LNCaP cells [135–137]. In parallel, phosphoinositide 3-kinase (PI3K) also plays a critical role in regulating the exocytosis of sPAcP [135]. Additionally, PKC activator and androgens both increase PAcP secretion, which are blocked by PKC inhibitors [138]. These results together suggest that sPAcP secretion is mediated by a regulatory process including Rab27a, PI3K, and PKC, differing from PSA secretion primarily in response to androgens [135, 138, 139].

Effects of Growth Factors on PAcP Expression

Factors other than androgens can regulate PAcP gene expression in LNCaP cells. Interestingly, the effect of EGF is more pronounced than DHT in determining PAcP mRNA expression and can potentiate the downregulation by androgens, but there is no added effect by androgens on EGF suppression [54, 70, 140]. EGF treatment also results in decreased cPAcP activity, which may be due to its phosphorylation inactivation by EGFR or oxidation inactivation [124]. While both EGF and TGF-α can bind to the EGF receptor [141, 142], TGF-α has a less inhibitory effect than EGF on reducing hPAcP mRNA [71]. On the other hand, TGF-β_1, which is inhibitory to normal prostatic epithelial cells [143, 144], upregulates the expression of PAcP mRNA [140]. Due to the importance of PAcP in clinical applications, further experiments should clarify the regulation of PAcP in prostate epithelia.

In the presence of androgens, the expression of growth factors, e.g., EGF, TGF-α, TGF-β_1, and TGF-β_3, are also modulated. Androgens and growth factors and their receptors, represent cross-talk at several levels. Additional experiments are required for elucidating the role of this cross-talk on the regulation of PAcP gene expression.

Epigenetic Regulation of PAcP Expression in Prostate Carcinoma Cells

DNA methylation and histone modification, two common epigenetic mechanisms, play vital roles in regulating PCa cell growth and metastasis [145]. Histone modification, primarily by acetylation and deacetylation, leads to altered gene expression by changing chromosome structure and the level of gene transcription. Histone

deacetylase (HDAC) activity is enhanced and upregulated in PCa and other carcinomas [146–148]. Hence HDAC is recognized as a promising target for cancer therapy, although the exact role of specific HDACs in the pathophysiology of PCa is still not well understood.

HDAC inhibitors have been shown to induce PCa cell growth arrest, differentiation, and apoptosis. cPAcP functions as a tumor suppressor in prostate carcinomas, and its decreased expression correlates with PCa progression. However, the molecular mechanism of its reduced expression in PCa remains an enigma [16, 19, 21]. Importantly, HDAC inhibitors, including sodium butyrate, trichostatin A (TSA), and valproic acid (VPA), suppress the growth of PCa cells and concurrently, cPAcP mRNA and protein expression are increased and ErbB-2 Tyr-P is decreased [149]. Conversely, knockdown of cPAcP expression by shRNA reduces the efficacy of HDAC inhibitor-induced growth suppression. Therefore, PAcP is involved in HDAC inhibitor-induced growth suppression and functions as a tumor suppressor gene in regulating PCa progression and metastasis (Fig. 12.3). Importantly, HDAC inhibitor-treated PCa cells increase their androgen responsiveness [149]. Understanding the regulation of cPAcP expression by HDACs may lead to improved CRPCa therapy by HDAC inhibitors.

Prostatic Acid Phosphatase as a Therapeutic Agent as Well as a Target for Prostate Cancer Treatment

While the majority of patients with metastatic prostate cancer have an initial response to ADT, most patients will eventually relapse with castration-resistant tumors. With the limited efficacy of conventional therapeutic approaches and also with significant morbidities of surgical and radiation treatments in advanced PCa, other avenues for treating advanced prostate carcinoma are actively under investigation.

PAcP Per Se as a Therapeutic Agent

Several lines of biochemical evidence have demonstrated that cPAcP functions as a tumor suppressor. A single intratumoral injection of an expression vector encoding the wild-type PAcP protein into xenograft tumors results in the suppression of tumor growth and progression [19]. In PAcP-knockout mice, the prostate develops carcinomas in situ, indicating that cPAcP functions as a tumor suppressor [20]. In parallel, cPAcP plays a critical role in HDAC inhibitor-induced PCa cell growth suppression [149]. Importantly, the HDAC inhibitor-treated PCa cells exhibit an increase in androgen responsiveness, suggesting that intermittent treatment with HDAC inhibitors may prolong the duration of ADT [149]. Thus, the restoration of cPAcP expression in PCa cells may provide a novel avenue for treating CRPCa.

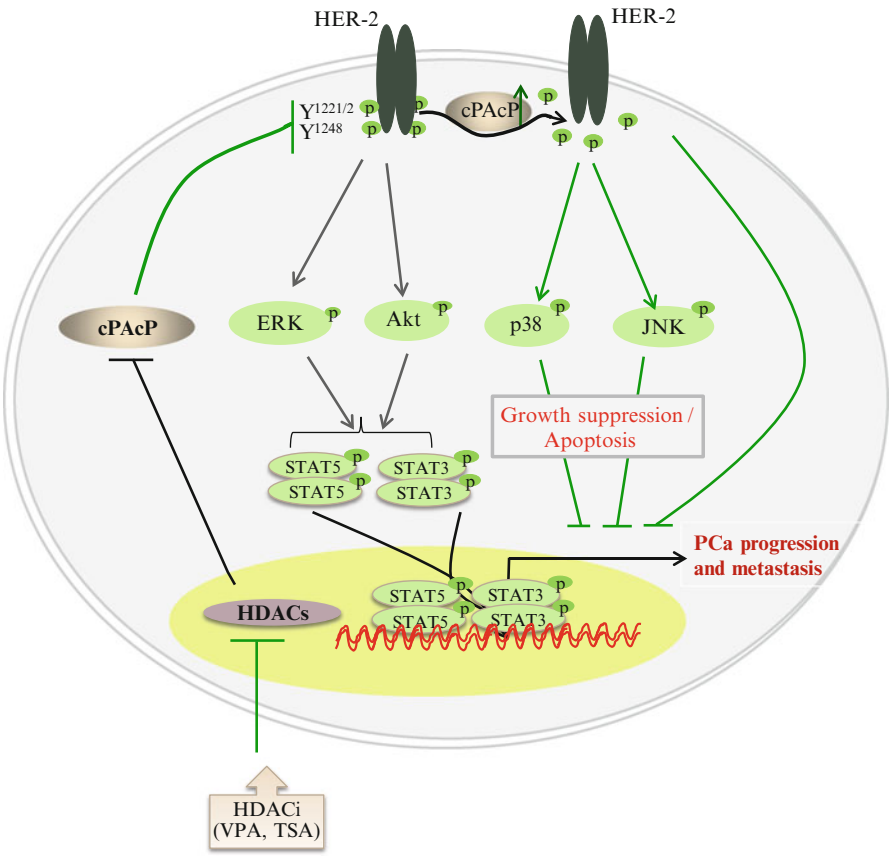

Fig. 12.3 Epigenetic regulation of prostate cancer cells. We propose that in PCa cells, upregulated histone deacetylases (HDACs) downregulate PAcP expression. This PAcP suppression leads to aberrant activation of ErbB2/HER-2/neu by Tyr-P followed by ERK/MAPK and Akt activation, leading to cell survival, proliferation, adhesion, and migration. Conversely, HDACs inhibitors (HDACi) restore cPAcP expression. This restored cPAcP dephosphorylates ErbB-2, which leads to inhibit prostate cancer progression and metastasis by p38 and JNK activation

PAcP as an Antigen for Immunotherapy of Prostate Cancer

Immunotherapeutic vaccines induce an antitumor response [150] by targeting tumor-associated antigens (TAAs) or by disrupting molecular pathways that promotes tumor growth [151, 152]. Therefore, the primary goal of immunotherapy is to activate the effector T cells that can migrate to the developing tumors and facilitate the damage of individual cancer cells.

Prostate cells express several specific biomarkers, including PSA, PAcP, and prostate-specific membrane antigen, which serve as TAAs and can serve as

immunogens. Previous studies demonstrated naturally occurring PAcP-specific binding IgG in human serum [65], the induction of a destructive prostatitis by PAcP-specific CTLs in rodents [153], and T-helper cell responses in men with PCa [154]. Studies have also identified Abs and circulating T cells against TAAs in PCa patients [155, 156]. These findings suggest that T-cells can break the tolerance and induce an immune response against tumor cells [155, 156]. These phenomena collectively indicate that an immune environment capable of supporting antigen-specific CTL may exist in vivo [154]. Small et al. [157] observed that dendritic cells loaded with an engineered antigen–cytokine fusion protein consisting of PAcP and granulocyte macrophage colony-stimulating factor (GM-CSF) are capable of inducing a potent cellular immune response in vivo to rodent tissues and tumors that express PAcP [157]. Subsequently, a dendritic cell product consisting of autologous dendritic cells loaded with the human PAcP–GM-CSF fusion protein was developed. It is hypothesized that when the vaccine is infused into the patient, the activated antigen-presenting cells (APCs) displaying the fusion protein will induce an immune response against the TAA. Phase I/II clinical trials with a dendritic cell-based PAcP vaccine in CRPCa patients led to a greater than 50 % decrease in PSA [157, 158]. The study showed that all patients developed specific immune responses to the recombinant fusion protein, and 38 % developed immune responses to PAcP. The time to disease progression correlated with the development of an immune response to PAcP and with the dose of dendritic cells received. There were minimal side effects of the therapy.

Highlight: Sipuleucel-T – An Autologous Dendritic Cell Product

Sipuleucel-T (Provenge, the commercial name) has become the first vaccine in the class of T cell-associated cancer immunotherapeutic agents approved by the United States Food and Drug Administration in April 2010 for the treatment of metastatic CRPCa. Sipuleucel-T is composed of autologous peripheral blood mononuclear cells (PBMCs), including APCs with a recombinant fusion protein PA2024 (full-length PAcP) linked to an adjuvant (GM-CSF). Currently, Sipuleucel-T is reserved for patients with documented metastatic PCa who have progressed on ADT with a documented testosterone level of less than 50 ng/dL. Sipuleucel-T treated patients demonstrated an additional 4.1-month median survival compared to the placebo group, which was statistically significant (HR = 0.78; 95 % CI, 0.61–0.98; $p = 0.03$). This increase in survival correlated with a 22 % decrease in mortality with the use of Sipuleucel-T [159, 160]. There is a need of surrogate markers for determining a patient's response to therapy. Clearly, the identification of predictive biomarkers will help practitioners select patients who are most likely to benefit from therapy [160].

Conclusion and Perspectives

Until the availability of PSA, circulating PAcP activity has served as a surrogate marker for diagnosing PCa and also has been used to monitor the efficacy of androgen deprivation therapy in treating PCa [14, 66]. In contrast, expression of PAcP and its intracellular level (cPAcP) is diminished in prostate carcinomas. Recent advances emphasize that cPAcP is an authentic protein tyrosine phosphatase and functions as a negative growth regulator in PCa cells. Importantly, cPAcP represents a novel subfamily of PTP super family [121]. The expression of PAcP is regulated at different levels as well as by different factors including androgens and growth factors in prostate carcinoma cells. The androgenic regulation of PAcP expression and secretion has been known to be a hallmark of androgen action for over six decades [2]. Nevertheless, the results of molecular studies demonstrate that the promoter activity of the PAcP gene is regulated in an androgen-independent manner [54, 126, 132].

Several lines of evidence support the importance of cPAcP enzyme in regulating PCa cell proliferation, particularly during the castration-resistant progression, at least in part by dephosphorylating p-Tyr of ErbB-2 intracellularly. It has been speculated that PAcP can also function as a phospholipid phosphatase because in cPAcP-knockdown cells, phospholipid activity is enhanced and also PAcP has an open active domain [80, 121]. Additionally, PAcP expression is in part regulated by epigenetic mechanism including histone acetylation and possibly methylation. These emerging data support PAcP as a potential therapeutic target for advanced PCa. The recent clinical immunotherapy trial with PAcP protein as a vaccine is promising. Further studies are needed to improve the clinical efficacy, for example, by effective intracellular delivery of antigenic peptides into dendritic cells.

Taken together, the data clearly show that cPAcP functions as an authentic tumor suppressor in PCa. Due to the importance of the PAcP gene in prostate carcinogenesis, investigation of the basic biochemistry and molecular biology of cPAcP including its interaction with other oncogenic proteins should provide valuable insights into its potential therapeutic applications.

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Part IV
Hormonal

Chapter 13
Truncated Androgen Receptor Splice Variants in Prostate Cancer

Peter E. Lonergan and Donald J. Tindall

Abstract The androgen receptor (AR) is fundamental for the growth and survival of normal and malignant prostate cells. Therefore, androgen deprivation therapy remains the first-line treatment for disseminated disease; however, relapse and progression to a castration-resistant phenotype for which no durable treatment currently exists, is inevitable. Restored AR activity is fundamental in the progression to castration-resistant prostate cancer. Multiple mechanisms by which AR is reactivated under androgen-depleted conditions may be involved in the development of this lethal phenotype. Recent studies have identified alternatively spliced transcripts encoding truncated AR isoforms that lack the ligand-binding domain, which is the therapeutic target of androgen deprivation therapy. Many of these truncated AR variants function as constitutively active, ligand-independent transcription factors that can support androgen-independent expression of AR target genes, as well as ligand-independent growth of prostate cancer cells. In this chapter, we will summarize the recent developments in the identification and characterization of AR splice variants in prostate cancer.

Androgen Receptor in Prostate Cancer

Prostate Cancer Incidence and Progression

Prostate cancer (PCa) is the most frequently diagnosed non-cutaneous malignancy in men and the second leading cause of male cancer-related mortality in

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the USA with an estimated 241,740 new diagnoses and 28,170 mortalities annually [1]. Clinically localized PCa is managed primarily through surgery or radiation therapy [2]. For patients who recur systemically following definitive treatment or who present with locally advanced or metastatic disease, the mainstay of treatment is androgen-deprivation therapy (ADT) typically with a luteinizing-hormone-releasing hormone (LHRH) agonist [3]. Progression-free and overall survival figures of patients with metastatic disease with various methods of ADT have ranged from 12 to 20 months and 24 to 36 months, respectively [4–7]. Progression to this lethal disease phenotype, heralded by rising serum prostate-specific antigen (PSA), increasing tumor size, new metastatic spread and disease-related symptoms is referred to as castration-resistant prostate cancer (CRPC; [8]).

Overview of AR Structure and Function

The human androgen receptor (AR) gene is a nuclear transcription factor and a member of the steroid hormone receptor superfamily of genes. It is located on the X chromosome (q11-12) and consists of eight exons. It encodes for a protein of 920 amino acids with a mass of 110 kDa. The structural organization of the AR gene, mature spliced mRNA, and protein domains are similar to other family members, including the estrogen receptor α (ERα), ERβ, and progesterone receptor [9, 10]. The AR consists of four structurally and functionally distinct domains: a poorly conserved N-terminal domain (NTD), a highly conserved DNA-binding domain (DBD), and a moderately conserved ligand-binding domain (LBD). A short amino acid sequence called the "hinge region" separates the LBD from the DBD and also contains part of a bipartite ligand-dependent nuclear localization signal (NLS) for AR nuclear transport [11, 12].

AR Exon 1

AR exon 1 encodes the entire NTD, which represents almost 60 % of the AR protein but is variable in length due to polymorphic (CAG)n and (GGN)n repeat units encoding polyglutamine and polyglycine tracts, respectively [13–15]. The AR NTD is a potent transcriptional activator and can activate transcription independently of androgenic stimulus in LBD deletion mutants [16, 17]. This activity has been mapped to two primary transactivation domains termed transactivation unit-1 (TAU-1) and TAU-5. These two domains are critical for full AR transcriptional activity [18]. TAU-1 activity has been more precisely mapped to a discrete ^{178}LKDIL^{182} motif [18, 19]. However, TAU-5 is responsible for the majority of constitutive transcriptional activity within the NTD, mediated through the core sequence ^{435}WHTLF^{439}, accounting for approximately 50 % aberrant AR activity in CRPC cells [20].

AR Exons 2–3

AR exon 2 encodes the first zinc finger in the AR DBD, which is the DNA recognition helix that makes contact with the major groove residues in an androgen-response element (ARE) half-site [21]. The second zinc finger is encoded by AR exon 3 which is the dimerization interface that mediates AR homodimer formation [21].

AR Exons 4–8

AR exons 4–8 encode a short 50 amino acid flexible hinge and 11 α-helices folded into an α-helical sandwich to form the well-characterized AR C-terminal domain (CTD), which contains the AR LBD and transcriptional activation function-2 (AF-2) coregulator-binding surface [22–26]. In the absence of ligand, the AR protein is localized to the cytoplasm, where it associates with HSP90, other molecular chaperones, and high-molecular weight immunophilins by virtue of interaction with the CTD [27, 28]. Androgen binding induces a conformational change in the AR, which exposes the bipartite NLS in the hinge region [29], thus allowing direct interaction with importin-α and subsequent nuclear localization through the nuclear pore complex [30]. In the nucleus, AR binds as a dimer to AREs in the promoter and enhancer elements of target genes, the best-characterized of which are PSA, TMPRSS2, NKX3.1, and hK2 [31]. Transcriptional activation of these target genes is a complex, multistep process that requires ordered-stepwise recruitment of a plethora of coregulatory proteins [12, 32]. Although the AF-2 domain is able to recruit well-characterized coactivators such as SRC-1, SRC-2/TIF-2, and SRC-3/AIB1, its contribution to transcriptional activity is relatively weak, compared to the AF-1 domain within the NTD [33].

Targeting AR in Prostate Cancer

Observations from as early as the 1780s noted that prostatic epithelium undergoes atrophy following castration [34]; however, it was the pioneering work of Huggins and Hodges in the 1940s which demonstrated that benign prostatic epithelium and prostate adenocarcinoma were biochemically analogous and respond in a similar fashion to androgen deprivation [35–37]. This principle of androgen deprivation is the standard-of-care for the clinical management of disseminated PCa. Currently, suppression of testicular androgen synthesis (which accounts for ~90 % of serum testosterone) can be effectively achieved with bilateral orchiectomy or medical castration with LHRH agonists [3]. This regime can be combined with direct AR antagonists such as bicalutamide, flutamide, or nilutamide; however, whether combined androgen blockade offers benefits beyond castration monotherapy remains controversial [38]. This therapeutic intervention leverages the extraordinary dependence of PCa cells on AR signaling and typically results in suppression of detectable

serum PSA, disease-related symptoms, and tumor regression. At the cellular level, both cell cycle arrest and cell death are observed upon inhibition of AR activity [39], thus underscoring the impact of AR as a valid therapeutic target in advanced disease. However, despite initially favorable results, disease progression to CRPC is inevitable, for which only a limited set of noncurative therapeutic options are available. Although multiple means exist through which CRPC cells evade AR-directed therapies, robust clinical and laboratory investigations support the hypothesis that the CRPC phenotype arises as a result of failure to suppress recurrent AR activity.

Recurrent AR Activity in Castration-Resistant Prostate Cancer

Radiological evidence of disease progression is invariably heralded by a rise in serum PSA, indicating that AR has been aberrantly reactivated despite ADT or combined androgen blockade. Investigating the means by which AR activity is restored in CRPC remains an area of intense investigation. To date four principal mechanisms have been identified in clinical specimens and validated in the laboratory, as playing key roles in disease progression.

AR Amplification/Overexpression

AR protein is expressed at high levels in most cases of CRPC (comparable to levels in untreated tumors); however, expression may be heterogeneous with a fraction of cells in some tumors being AR-low or -negative [40–43]. High levels of AR expression are also independently predictive of increased risk of death from PCa [44]. Consistent with immunohistochemical data, AR mRNA is also highly expressed in CRPC, with levels being several-fold higher than in primary untreated tumors [45–47]. One mechanism for increased AR mRNA expression is AR gene amplification, which occurs in about one-third of CRPC cases [48, 49]. In addition, there is continued expression of AR-regulated genes (such as PSA and also TMPRSS2:ERG fusion genes), indicating that AR transcriptional activity becomes reactivated in CRPC [40, 45, 46]. A landmark study by Chen and colleagues demonstrated that resurgent AR activity after castration of the host is sufficient to confer the CRPC growth phenotype in vivo using xenograft models of PCa [50].

AR Mutations

A number of AR mutations, primarily within the LBD, have been identified in CRPC that can enhance AR activation by weak adrenal androgens and other steroid hormones such as progesterone, estradiol, and cortisol [47, 51]. The use of AR antagonists can further select for mutations that convert these therapeutic agents into agonists [52]. The most well studied of these mutations at codon 741 (W741C and W741L) identified in LNCaP cells after long-term culture with bicalutamide, has also been identified in CRPC patients [53–55]. However, the overall frequency

of AR mutations in patients treated initially with ADT is quite low, and this is unlikely to be a major mechanism for progression to CRPC [54]. Nonetheless, a recent assessment of AR mutations in circulating tumor cells revealed AR mutations in 20 out of 35 patients with CRPC, indicating that the frequency of alteration could be even higher in cells with metastatic potential [56].

Intracrine Androgen Production

Another mechanism that may contribute to AR activation following castration is the upregulation of enzymes that convert weak adrenal androgens to testosterone at sufficient levels to restore AR activity [46, 57, 58]. Adrenal-derived dehydroepiandrosterone (DHEA) can be converted to testosterone and dihydrotestosterone (DHT) through the action of tumor-derived 3β-hydroxysteroid dehydrogenase (3β-HSD) type 1 and type 2 (HSD3B1, HSD3B2), type 5 17β-HSD (AKR1C3) and SRD5A2 [59–62]. Significantly, HSD3B2, AKR1C3, SRD5A1, AKR1C2, and AKR1C1 are often altered in CRPC compared to primary tumors, lending support to the hypothesis that intracrine androgen synthesis plays a role in CRPC [46, 63–65]. Therefore, targeting the intracrine androgen synthesis pathway has led to the use of CYP17,20-lyase inhibitors such as abiraterone, which have shown particular promise for the treatment of CRPC [66].

AR Cofactor Alteration and Post-translational Modifications

Post-translational modifications of AR have been suggested to contribute to enhanced AR function in CRPC, including serine/threonine phosphorylation [67], tyrosine phosphorylation [68, 69], acetylation [70], ubiquitylation [71, 72], and sumolyation [73]. However, while many of these events are known to occur downstream of growth factor pathways, it remains uncertain whether therapeutically targeting these growth factor receptors or downstream kinases are of clinical benefit. Finally, an extensive body of literature [32] suggests that AR cofactors may be deregulated in CRPC, including upregulation of coactivators [74], loss of corepressors [75], or AR mutations that alter the way that cofactors are perceived [76]. However, the simultaneous involvement of multiple coregulators and their overlapping interaction suggest that additional studies are required to determine the full contribution of coregulators in aberrant AR activity in CRPC.

Identification of AR Variants

Truncated AR Variants in Benign and Non-prostate Tissues

In addition to the wild-type AR protein (110 kDa), some lower molecular-weight protein bands are expressed in some AR-expressing cell lines, which are

immunoreactive with an antibody raised against an epitope mapping to the AR NTD. The origin of these short AR isoforms was quite controversial and at least four potential mechanisms underlying their generation were proposed: (1) alternative translation start codons, (2) proteolytic cleavage, (3) premature stop codon resulting from mutation, and (4) alternative transcription start site.

AR-A

In 1993, Zoppi and colleagues identified a mutation in the AR NTD carried by a 46,XY phenotypic female (subject R776) with the syndrome of complete testicular feminization [77]. This mutation, termed R776, introduces a premature stop codon in place of the normal AR codon for amino acid 60. COS cells transfected with cDNA encoding the R776 mutation produces an 87 kDa form of AR that lacks the normal NTD. Although this short AR isoform can activate an androgen-responsive reporter gene coexpressed in CV1 cells, the level of activation is markedly reduced when compared to the effect of full-length AR in this system [77]. It was proposed that the 87 kDa form (termed AR-A) is due to translation initiation of AR protein at the internal methionine 188 residue of the full-length AR [78]. They also suggested that AR-A and full-length AR may differ in their ability to activate target genes and are regulated differently in various cell types [78].

AR45

AR45 is a naturally occurring truncated AR variant originally identified by 5′ rapid amplification of cDNA ends (RACE) with RNA isolated from human placental tissue [79]. The AR45 transcript was found to originate from alternative splicing of a previously unreported exon located ~22.1 kb downstream of AR intron 1. This cryptic exon, termed exon 1B can be spliced in place of AR exon 1 to yield a 45 kDa isoform containing the DBD, CTD, and a novel seven amino acid sequence at the NH_2 terminus in place of the wild-type AR NTD. Analysis of AR45 by RT-PCR revealed expression in a number of human tissues with highest levels in heart and skeletal muscle tissue. However, these RT-PCR analyses were not quantitative, so it is unclear how AR45 expression levels compare with full-length AR in these tissues. It is also unclear whether AR45 transcript is translated into endogenous protein in these tissues; however, immunoblot analysis of LNCaP lysates demonstrated the presence of a ~45 kDa isoform that was immunoreactive with an antibody specific for the AR CTD. AR45 overexpressed in CV-1 cells was shown to bind androgen, localize to the nucleus, interact with the full-length AR NTD, and inhibit full-length AR activity in a ligand- and DBD-dependent manner. Ectopically expressed AR45 in LNCaP cells also inhibited proliferation. These observations suggest that AR45 is a negative regulator of AR signaling. However, when the coactivator TIF2 or the oncogene β-catenin was overexpressed, AR45 stimulated androgen-dependent promoters in the presence of androgens [79]. Interestingly,

AR45 has been implicated in an increased risk of drug-induced cardiac arrhythmia, long QT syndrome in women. The Human Ether-a-go-go-Related Gene (HERG) potassium channel is largely responsible for determining QT interval, and androgen-mediated stabilization of HERG protein was observed in cells transfected with AR45 but not full-length AR [80].

AR Splicing in Androgen Insensitivity Syndrome

Androgen insensitivity syndrome (AIS) is an X-linked condition first described in 1974 in 46,XY individuals as a result of AR genetic alterations characterized by end-organ resistance to androgens, leading to defects in male sexual differentiation [81]. These AR alterations result in AR proteins with impaired function that prevents normal androgen signaling and development of internal and external male phenotypic characteristics [82]. The most severe AIS phenotype is complete AIS (CAIS), in which individuals have female external genitalia, a short, blind-ending vagina, the absence of Wolffian duct-derived structures such as the epididymes, vas deferens, and seminal vesicles, the absence of a prostate, development of gynecomastia, and the absence of pubic or axillary hair [83]. Partial AIS (PAIS) includes a wide range of phenotypes including a predominantly female appearance, ambiguous genitalia, and a predominantly male phenotype [83]. In general, the severity of AIS is proportional to the level of impaired AR activity caused by specific alterations in the AR gene. Several types of AR mutation have been identified in individuals with AIS, including (1) single point mutations resulting in amino acid substitutions or premature stop codons, (2) nucleotide insertion/deletion leading to frame shirt and premature termination, and (3) complete or partial gene deletion [84]. Interestingly, a number of alternatively spliced AR transcripts have been identified in AIS leading to AR proteins with impaired function. AR gene defects, often within intronic splice donor or splice acceptor sites, have been implicated with these alternatively spliced variants.

AR-Splicing Disruption in Complete AIS

A number of alternatively spliced AR variants have been described in patients with CAIS. A G→T point mutation within the splice donor site of intron 4 has been described in a patient with CAIS [85]. This mutation was shown to lead to the use of an alternative cryptic splice donor in exon 4, resulting in an in-frame 123 bp deletion from the AR transcript and a concomitant 41 amino acid internal deletion of the AR protein. This variant displayed no androgen-binding activity and no transcriptional activity on an androgen-responsive promoter gene. Similar mutations in splice donor sites of introns 2 and 7 have been reported in two other individuals with CAIS, leading to skipping of exons 2 and 7 from AR transcripts, respectively [86, 87]. The exon 2-deleted AR protein was prematurely truncated as a result of a premature stop codon encoded by an out-of-frame exon 3 [87]. The exon 7-deleted AR protein was ~98 kDa [86]. In both these studies, the AR variants identified were unable to bind androgens.

AR Splicing Disruption in Partial AIS

Alternatively spliced AR variants have also been identified in individuals with PAIS. This suggests that cells with altered AR splicing can retain some residual AR activity. This may result from AR variants maintaining some degree of transcriptional activity or a residual level of splicing that permits synthesis of the full-length AR transcript and protein. A >6 kb in-frame deletion has been reported in AR intron 2 in a patient with PAIS, which results in skipping of exon 3 and synthesis of an AR variant protein with a deletion of 39 amino acids from the DBD [88]. This AR variant exhibited normal androgen binding, but no transcriptional activity on an androgen-responsive promoter gene. A T → A mutation 11 bp upstream of exon 3 has also been described and has been shown to be associated with two alternatively spliced AR transcripts [89]. The first alternatively spliced AR transcript arose through skipping of exon 3, and the other resulted from a cryptic splice acceptor site 71 bp upstream of exon 3. The AR protein encoded by this alternatively spliced acceptor site contained an additional 23 amino acid in-frame insert in the DBD between the two zinc fingers [89]. Functionally, this receptor was shown to bind androgens, but no DNA-binding activity was observed in a band shift assay. In another PAIS individual, a G → T mutation in the splice donor site of intron 6 resulted in expression of a larger AR transcript species [90]. An in-frame stop codon 79 bp downstream from the donor splice site in intron 6, resulted in a smaller, truncated AR protein. Impaired androgen binding was observed in this AR variant [90]. Finally, an additional splicing mutation was identified as a C → T mutation in codon 888 of exon 8 with replacement of the final 33 amino acids of the full-length AR protein with a novel eight amino acid sequence as a result of a cryptic splice donor site [91]. Functionally, this AR variant exhibited impaired androgen-binding capacity and no transcriptional activity on an androgen-responsive promoter gene.

Truncated AR Variants in Malignant Prostatic Tissues

AR alternative splicing can give rise to CTD-truncated AR protein isoforms that lack the AR LBD in PCa cells. These AR isoforms are lacking the functional domain of the receptor that would be predicated to mediate responses to traditional ADT, as well as new AR-targeted therapies such as abiraterone [66, 92], enzalutamide (formerly MDV3100; [93, 94]), and emerging next-generation anti-androgens such as ARN-509 [95].

Identification of AR Variants in the CWR22 Model

Seminal observations leading to the discovery of alternatively spliced truncated AR variants were initially reported in the 22Rv1 cell line [96]. 22Rv1 cells are derived

from the CWR22 transplantable human prostate tumor model [97]. This tumor exhibits androgen-dependent growth and PSA secretion in male nude athymic mice and regresses in response to androgen withdrawal as defined by tumor shrinkage and a decline in serum PSA levels [98]. Importantly, it simulates the clinical course of PCa; in that relapsed growth of the tumor (designated CWR22R) occurs after several months of androgen withdrawal and is preceded by rising PSA levels. The CWR22Rv1 cell line (also termed 22Rv1) was established from the relapsed CWR22R tumor serially propagated in mice [99] and is characterized by androgen-independent proliferation, AR expression, and androgen responsiveness. It serves as a model possessing an intermediate phenotype between that of hormone-sensitive, AR-positive cells lines (e.g. LNCaP) and androgen-independent, AR-negative cells lines (e.g. DU-145 and PC-3).

ARΔLBD

In 2002, Tepper and colleagues made the critical observation that 22Rv1 cells express two discrete AR protein isoforms of 110–114 kDa and 75–80 kDa [96]. Using an antibody mapping approach, it was demonstrated that the smaller 75–80 kDa AR species contained an intact transactivation domain and DBD but lacked the LBD (referred to as ARΔLBD). Further biochemical characterization determined that the ARΔLBD isoform was constitutively nuclear and could bind DNA independent of androgens. Co-immunoprecipitation experiments indicated that the ARΔLBD isoform did not interact with full-length AR, suggesting that this species functioned as an independent transcription factor [96]. In addition, characterization of the 22Rv1 full-length AR transcript using RT-PCR mapping with overlapping primer pairs revealed an insertion of an in-frame 117 bp sequence occurring between the 3′-end of exon 3 and the 5′-end of exon 4, which was identified as AR exon 3. This represents a tandem duplication, referred to as exon 3′ and encodes an additional 39 amino acids and 4–5 kDa of protein mass, thus accounting for the observed increase in size of 22Rv1 full-length AR. Exons 2 and 3 each encode zinc fingers comprising the DBD. Therefore, the 114 kDa 22Rv1 full-length AR species contains an additional zinc finger [96]. Interestingly, these mutations were not detected in the original androgen-dependent CWR22 xenograft, indicating that this change occurred during the progression to androgen independence.

Several recent studies have indicated that alternative splicing may be an important contributor to the synthesis of truncated AR species lacking the LBD in 22Rv1 and other cells. This concept was first suggested by Dehm and colleagues in 2008 based on the observation of differential siRNA targeting of the various AR isoforms in 22Rv1 cells [100]. siRNA targeted to AR exons 7/8 abolished expression of full-length AR in 22Rv1 cells but had no effect on the smaller 75–80 kDa isoform. Conversely, siRNA targeted to AR exon 1 knocked down expression of both AR isoforms in 22Rv1 cells. Functionally, androgen-dependent expression of AR target genes and cell proliferation were shown to be attributable to full-length AR, whereas androgen-independent expression of AR target genes and constitutive,

androgen-independent cell proliferation were shown to be supported by the smaller 75–80 kDa isoforms. These important observations strongly suggested that different mRNA species encode the different AR protein species in 22Rv1 cells and provided the foundation for 3′ RACE and other approaches to identify their origin [100–107]. This led to the identification of a series of alternatively spliced AR transcripts expressed in 22Rv1 cells that result from cryptic exons located within introns 2 and 3.

AR 1/2/2b and AR 1/2/3/2b

AR exon 2b was identified following 5′ RACE experiments with RNA derived from 22Rv1 cells [100]. AR exon 2b was shown to splice downstream of either AR exon 2 or AR exon 3, resulting in truncated AR proteins containing the AR NTD, one or both zinc fingers of the DBD, and a short 11 amino acid COOH terminus extension encoded by AR exon 2b (Fig. 13.1; Table 13.1). Functionally, AR 1/2/2b and AR 1/2/3/2b exhibited constitutive transcriptional activity on promoter–reporter gene assays. The AR 1/2/2b transcript was also shown to be expressed in VCaP cells as well as the LuCaP 23.1 and 35 xenograft models of PCa progression.

AR-V1 to AR-V7

A comprehensive bioinformatics analysis of the ~170 kb AR intron sequences against the National Center for Biotechnology Information (NCBI) human expressed sequence tag database was performed by Hu and colleagues to identify “intronic” genomic fragments [102]. Using this approach, three cryptic exons were identified in AR intron 3 (termed CE1, CE2, and CE3) and one in AR intron 2 (termed CE4). In order to determine the exon–intron splicing junctions, RT-PCR was used to amplify and sequence transcripts containing AR exon 2 and the putative cryptic exons in 22Rv1 cells. Sequencing of the amplicons defined the 5′ junctions of CE1, CE2, and CE3 and the 5′ and 3′ junctions of CE4 and was used to construct seven AR variant transcripts, named AR-V1 to AR-V7. The genomic coordinates of CE4 are identical to the novel exon AR 2b identified by Dehm and colleagues [100]. Significantly, all seven AR variants harbor premature stop codons downstream of AR exon 2, generating LBD-truncated AR proteins.

Two alternatively spliced AR isoforms identified in this study were further characterized. AR-V1 (composed of AR exons 1/2/3/CE1) was detected by quantitative RT-PCR at greater levels in RNA isolated from CRPC specimens compared with hormone naïve primary PCa specimens [102]. AR-V7 (composed of AR exons 1/2/3/CE3) transcript was detected in various clinical specimens with the highest levels in CRPC tissues. Interestingly, a subset of hormone-naïve primary PCa specimens expressed AR-V1 and AR-V7 transcript at levels comparable to those CRPC specimens. Elevated AR-V7 mRNA expression in these 66 patients was associated with a poor clinical outcome as defined by biochemical recurrence following

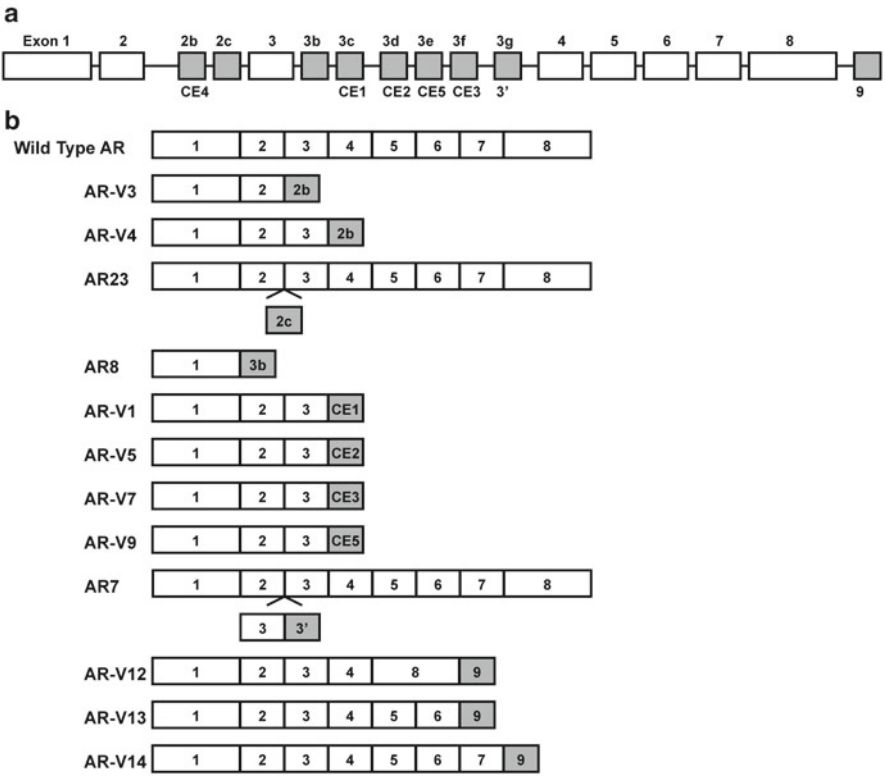

Fig. 13.1 Alternatively spliced AR isoforms identified in prostate cancer. (**a**) Schematic representation of the AR gene locus with locations of alternatively spliced, cryptic exons in *gray* (depictions are not to scale). Nomenclature of novel exons is based on the relative position within the AR locus. (**b**) Alternative splicing of cryptic exons in the AR locus or exon skipping gives rise to C-terminal truncated AR transcript isoforms. Additional alternatively spliced mRNA isoforms which have not been cloned or characterized are not illustrated

prostatectomy. In this same cohort, higher levels of full-length AR or AR-V1 transcript were not associated with biochemical recurrence [102].

AR3 to AR5

In a separate study, Guo and colleagues employed the CWR-R1 cell model to identify novel AR splicing variants [101]. The CWR-R1 cell line is derived from recurrent CWR22 xenograft tumors harvested from male nude mice 140–160 days after castration [109]. Expression of AR using an antibody recognizing the AR NTD revealed the full-length AR at 110 kDa and also an 80 kDa immunoreactive band. Using a panel of shRNAs targeting distinct regions of the AR gene suggested that both AR isoforms may be translated from more than one transcript. Using 3′ RACE

Table 13.1 Human AR splice variants

taVariant	Alternate names	Intron retained/ novel exon	Splicing junction	Exons excluded	Unique COOH terminus peptide	Localization	Activity	References
AR23	–	2c	2/2c	–	23 inserted in DBD	Cytosolic	Conditional	[108]
AR8	–	3b/3′	1/3′	2–8	33	Cytosolic	–	[107]
AR-V1	AR4	3c/CE1	3/CE1	4–8	19	Cytosolic	Conditional	[101, 102]
AR-V2	–	3c/CE2	3/3/CE1	4–8	19	–	–	[102]
AR-V3	AR 1/2/2b, AR6	2b/CE4	2/CE4	3–8	11	–	Constitutive	[100–102]
AR-V4	AR1/2/3/2b, AR5, ARV6	2b/CE4	3/CE4	4–8	11	Nuclear	Constitutive	[100–102, 104]
AR-V5	–	3d/CE2	3/CE2	4–8	1	–	–	[102]
AR-V6	–	3d/CE2	3/CE2	4–8	6	–	–	[102]
AR-V7	AR3	3f/CE3	3/CE3	4–8	16	Cytosolic/Nuclear	Constitutive	[101, 102]
ARV7	–	3g/3′	3/3/3′	4–8	22	Nuclear	Constitutive	[104]
AR-V8	–	3	3/intron 3	4–8	10	–	–	[103]
AR-V9	–	3e/CE5	3/CE5	4–8	23	Cytosolic	Conditional	[103, 106]
AR-V10	–	3	3/intron 3	4–8	39	–	–	[106]
AR-V11	–	3	3/intron 3	4–8	20	–	–	[106]
ARV567es	AR-V12	9	4/8/9	5–7	10	Nuclear	Constitutive	[103, 105]
AR-V13	–	9	6/9	7–8	3	–	Inactive	[103]
AR-V14	–	9	7/9	8	7	–	–	[103]

with a primer corresponding to a target sequence in AR exon 1, more than 20 splicing variants were identified in CWR-R1 cells with three variants (designated AR3, AR4, and AR5) predicted to encode a protein ~80 kDa [101]. The splicing patterns for AR3 and AR4 transcripts were identical to AR-V7 (AR exons 1/2/3/CE3) and AR-V1 (AR exons 1/2/3/CE1) identified by Hu and colleagues, respectively, in 22Rv1 cells [102]. Similarly, the splicing configuration of AR5 was identical to AR 1/2/3/2b and AR-V4 (AR exons 1/2/3/CE4) both identified independently in 22Rv1 cells [100, 102].

Of particular significance in this study, the authors developed an antibody against the unique 16 amino acid sequence at the COOH terminus of AR3 and demonstrated that protein expression of this isoform was increased in CRPC versus hormone-naïve PCa in a human PCa tissue microarray [101]. Also, cytoplasmic expression of this isoform could predict biochemical recurrence following prostatectomy [101]. Interestingly, the AR3 isoform was also expressed at the transcript and protein levels in benign prostate tissue, indicating that there may also be a role for this splicing event in normal cells. Functionally, AR3 was demonstrated to function as a constitutively active, ligand-independent transcription factor and could induce a CRPC growth phenotype in LNCaP cells grown in vitro and as xenografts in vivo [101]. Overall, this body of work demonstrates that an AR variant derived from contiguous splicing of AR exons 1/2/3/CE3, termed AR3 or AR-V7, is a clinically validated AR isoform that could play an important role in supporting the CRPC phenotype.

ARV6 and ARV7

Additional alternatively spliced AR isoforms have also been cloned from 22Rv1 cells using a yeast functional assay to identify AR cDNAs with constitutive or ligand-induced transcriptional activity [104]. This approach led to the identification of an additional cryptic exon in AR intron 3 termed 3′. Sequencing of the isolated cDNA, which was named ARV7 in this study, demonstrated that exon 3′ spliced downstream of the tandem-duplicated exon 3 in 22Rv1 cells. This 98 bp intronic insert resulted in a novel stop codon, giving rise to a truncated isoform containing the entire AR NTD, DBD, an extra zinc finger encoded by the extra copy of exon 3 and a novel 22 amino acid COOH terminus extension encoded by exon 3′. The authors also describe an additional AR splicing isoform in 22Rv1 cells, termed AR6, which exhibits an intronic 280 bp insert that is identical to the 2b exon first reported by Dehm and colleagues [100]. However, this intronic insert is downstream of the first AR exon 3, followed by the complete remaining AR exons 4–8 and also exhibits a trinucleotide (TAA) deletion. The polyadenylation signal described by Dehm and colleagues is present in the intronic sequence but has no influence on mRNA transcription termination. However, ARV6 mRNA translation results in a truncated AR isoform from an intronic novel stop codon further downstream [104]. The reason for this discrepancy is not clear, but could be due to the fact that 22Rv1 cells harbor two genomic copies of AR exon 2b [110].

AR8

The novel AR isoform AR8 was identified in CWR-R1 cells using 3′ RACE [107]. This isoform is composed of AR exons 1, 3, and 3b (identical to CE3). Due to usage of an alternative splice acceptor site in exon 3 (denoted as 3′), this transcript is deduced to encode a protein containing the NTD and a unique 33 amino acid sequence at the COOH terminus. AR8 transcript was detected in a panel of benign and malignant tumor specimens. The level of AR8 transcript was also elevated in the castration-resistant LNCaP sublines, C4-2 and C4-2b, as well as CWR22 xenograft tumors compared to their androgen-dependent counterparts. Using a polyclonal antibody specific for the unique COOH terminus of the AR8 protein, endogenous AR8 protein expression was detected in a number of castration-resistant PCa cell lines including CWR-R1, VCaP, C4-2, and C4-2b [107].

Identification of AR Variants in the VCaP Model

The VCaP cell line has been an additional cell-based model for studying alternatively spliced AR variants, following the observation of a smaller 75–80 kDa AR species that is immunoreactive with antibodies directed to the NTD, but not the LBD [100]. VCaP cells are derived from PCa tissue harvested at autopsy from a metastatic lesion to a lumbar vertebral body of a patient with CRPC, aseptically xenografted into SCID mice, and later harvested and plated onto tissue culture dishes [111].

AR-V8 to AR-V11

Watson and colleagues employed an adaptation of the 3′ RACE method whereby 3′ RACE products produced using a forward primer spanning the AR exon 2/3 junction in VCaP cells were sequenced using two next-generation sequencing platforms, 454 and SOLiD [106]. This approach confirmed splicing of AR exons CE1 and CE3 downstream of AR exon 3 and also identified four additional isoforms, named AR-V8 through AR-V11. These novel AR transcripts resulted from AR exon 3 read-through, splicing of novel cryptic exons in AR intron 3, or use of a different splice acceptor site in exon CE3. As in previous studies, these novel AR mRNAs would be predicted to encode variable-length COOH terminus extensions following the exon 3 sequence. Using this approach, additional novel AR transcripts were identified in VCaP cells that appear to result from exon skipping (AR exons 4/6, 4/7, 4/8, and 6/8) and splicing of a novel cryptic exon within AR intron 5 [106]. However, no full-length cDNAs were isolated from VCaP cells that correspond to these newly identified AR transcripts, so it is unclear whether these novel AR isoforms represent splicing intermediates, splicing errors, or are actual alternatively spliced AR transcripts that encode functional proteins.

Identification of AR Variants in the Mouse Myc-CaP Model

AR variant discovery has also been extended into murine models of PCa using the Myc-CaP model. The Myc-CaP cell line is derived from tumors that arose in mice with a prostate-specific c-myc transgene, which drives carcinogenesis in a stepwise fashion from prostatic intraepithelial neoplasia to invasive cancer [112, 113].

mAR-V1 to mAR-V4

Watson and colleagues employed a 3′ RACE approach combined with next generation sequencing in the Myc-CaP model and demonstrated the synthesis of four novel alternatively spliced AR isoforms, designated mAR-V1 to mAR-V4 [106]. mAR-V1 is composed of contiguously spliced mouse exons 1/2/3 followed by read-through into intron 3, with a unique 40 amino acid COOH terminus sequence before an in-frame stop codon. Unlike the human AR variants already described, mAR-V2 and mAR-V4 were found to harbor novel exons that map outside the mouse AR gene to distal regions on the X chromosome. The first exon, located ~250 kb downstream of the AR locus is spliced after AR exon 3 in the mAR-V2 variant. The other exon, located ~1 Mb upstream of the AR locus is spliced after AR exon 4 to yield mAR-V3 and mAR-V4. The structural basis for the generation of these isoforms at the genomic level remains unclear. However, the fact that Myc-CaP cells have AR gene amplification raises the possibility of intrachromosomal gene rearrangements.

Protein expression of mAR-V2 and mAR-V4 and isoform-specific knockdown in Myc-CaP cells confirmed these species [106]. Functionally, mAR-V4 is constitutively transcriptionally active in a ligand-independent manner in an androgen-responsive promoter–reporter assay and is also localized to the nucleus. However, mAR-V2 does not display transcriptional activity and is localized to the cytoplasm. Consistent with these findings, LNCaP xenografts stably expressing mAR-V4, but not mAR-V2 supports tumor growth in castrated mice.

Identification of AR Variants in the LuCaP Xenograft Model

The LuCaP series is an extensive panel of human PCa xenograft models derived from a range of primary PCa, soft tissue, lymph node, and osseous bone metastases (obtained during surgery or during a rapid autopsy) developed at the University of Washington, Seattle. These xenograft models have proved to be valuable resource for furthering our understanding of PCa progression from an androgen-dependent phenotype to a CRPC phenotype.

ARV567es

Sun and colleagues performed a comprehensive RT-PCR analysis on a panel of 25 LuCaP xenografts, mostly derived from metastases obtained from men with CRPC after prolonged ADT [105]. Two of the LuCaP xenografts, 86.2 and 136 (derived from a bladder metastasis and cells from ascitic fluid, respectively, from two different men who had relapsed following prolonged ADT) express shorter AR transcripts in the region spanning AR exons 2–8. Sequencing the short AR transcripts from both xenografts found identical cDNA sequences, lacking AR exons 5, 6, and 7, hence the designation ARV567es, in which "es" denotes exons skipped [105]. While the full nucleotide sequence of AR exon 8 is present, due to the splicing of exon 4 to exon 8, a frame-shift occurs in the open reading frame of ARV567es, resulting in a premature stop codon after the first 30 nucleotides, and leading to a shortened AR exon 8 sequence of 10 amino acids. Interestingly, in the LuCaP 86.2 xenograft, neither full-length AR transcript nor protein appears to be expressed which is in contrast to 22Rv1 and other models where full-length AR expression remains high. Using primers designed to specifically detect the AR exon 4–8 junction present in ARV567es, RT-PCR detected this AR variant isoform in patient specimens of primary PCa, CRPC, as well as benign prostate epithelium [105]. Functionally, AR567es acts as a constitutively active, ligand-independent transcription factor that supports CRPC cell growth in vitro and in vivo [105].

Identification of AR Variants in CRPC Specimens

AR-Q640X

AR-Q640X is a nonsense mutation that results in a substitution of the glutamine residue in AR position 640 by a premature stop codon identified using a yeast-based functional assay on metastatic bone marrow aspirate from a man who had progressed on ADT [114]. This mutation was subsequently identified in other CRPC patients [115]. This AR isoform was immunoreactive at ~75 kDa with an AR NTD-directed antibody confirming that the Q640X mutation led to a CTD truncated protein [114]. The AR-Q640X mutation is located downstream of the NLS within the second zinc finger of the DBD and the hinge region; therefore, a nonsense mutation at this codon may be predicted to confer constitutive transcriptional capabilities. Consistent with this hypothesis, AR-Q640X displays constitutive transcriptional activity in a ligand-independent manner in an androgen-responsive promoter–reporter assay and is also constitutively localized to the nucleus [114].

AR23

The functional yeast assay approach was also used to detect an AR splice variant named AR23, from a bone metastasis in a man with CRPC [108]. This AR isoform results from an exon/intron splicing error resulting in a 69 bp insertion which corresponds to the 3′-part of AR intron 2. The insertion occurs at the junction of exon 2 and exon 3, which encode for the first and the second zinc finger, respectively. Analysis of this novel AR variant cDNA sequence revealed no mutation in the classical donor or acceptor splice sites. As a result of this aberrant splicing, the last 69 bp of intron 2 persist in AR transcripts. These supplementary nucleotides do not lead to a premature stop codon but rather give rise to an AR variant, containing an insertion of 23 amino acids between the two zinc fingers of the DBD [108]. Functionally, AR23 remains in the cytoplasm and nuclear translocation is impaired following androgen binding. Despite the predicted lack of DNA-binding function, expression of AR23 in LNCaP cells increased transcriptional activity at androgen-responsive promoters. The mechanism remains unclear; however, AR23 was able to stimulate NF-κB transcriptional activity and decrease AP-1 transcriptional activity, suggesting that non-genomic functions of novel AR splice variants may be important in PCa disease progression [108].

AR-V12 to AR-V14

A novel method has identified additional alternatively spliced AR isoforms in CRPC specimens based on selective linear amplification of sense RNA (SLASR) using an AR exon 3-anchored forward primer, followed by detection of amplified transcripts using an AR gene tiling array [103]. Using this approach, the synthesis of mRNAs containing AR exons 2b/CE4, CE1, CE2, and CE3 were confirmed in 22Rv1 cells along with a novel isoform termed AR-V9, which was produced by contiguous splicing of AR exons 1/2/3 and a novel cryptic exon located in AR intron 3, termed CE5. This AR isoform is identical to the AR-V9 isoform reported in VCaP cells [106]. This novel approach was then deployed in RNA derived from two CRPC specimens, leading to the identification of an additional novel AR exon, termed "exon 9." This exon is located downstream of AR exon 8 and was shown to be the most 3′ exon in three novel AR mRNA species, termed AR-V12 (AR exons 1/2/3/4/8/9, which encodes the ARV567es variant), AR-V13 (AR exons 1/2/3/4/5/6/9), and AR-V14 (AR exons 1/2/3/4/5/6/7/9; [103]). Functional characterization of AR-V9 and AR-V12 elucidated varying degrees of constitutive ligand-independent transcriptional activity depending on the cell line studied. AR-V9 functions independently of full-length AR in LNCaP cells. AR-V9 and AR-V12 transcript expression is significantly elevated in CRPC specimens compared to hormone-naïve and benign specimens. Follow-up data available for patients with

hormone-naïve PC found no association between AR-V9 or AR-V12 expression and biochemical recurrence. Moreover, neither AR-V9 nor AR-V12 was associated with preoperative PSA values or disease stage. However, significantly higher AR-V12 expression levels, but not AR-V9, were found in patients with Gleason score 8 and above, when compared to those with Gleason score 6 and below or Gleason score 7 [103].

Characterization of AR Variants

Function of AR Variants

A particularly intriguing observation made by several groups is that although AR variants are expressed at highest levels in CRPC specimens, AR variants are expressed at variable levels in benign prostate epithelium, as well as hormone-naïve primary PCa specimens [101–103, 105]. In one study, full-length AR was detected in 35 out of 36 samples of laser captured microdissected (LCM) benign prostate biopsies from men (aged 35–55) without PCa and serum PSA less than 2 ng/ml who were administered ADT as part of a contraception study. In this study, a total of ten samples (27.8 %) also expressed ARV567es or AR-V7 [105]. In a separate cohort of non-castrate men (ages 59–70) who had radical prostatectomy for PCa, analysis of paired LCM benign and malignant prostate epithelial samples revealed ARV567es and AR-V7 transcript expression in benign, malignant, or both specimens [105]. These data suggest that AR variants are not necessarily etiologic in PCa initiation but may serve to facilitate progression when ADT is applied. This phenomenon has been demonstrated using LNCaP cells stably expressing ARV567es [105], VCaP [106], LuCaP 35 [105, 106], and LuCaP 136 [105] xenografts grown in castrate versus intact SCID mice.

Another hypothesis is that AR variant expression may be a mechanism of resistance to current ADT therapies and development of CRPC. Overexpression of AR-V7 in LNCaP cells and specific siRNA-targeted knock down of endogenous AR variants in 22Rv1 cells result in increased and decreased growth respectively, under CRPC-like condition in vivo and in vitro [100, 101, 106]. ARV567es increases proliferation of LNCaP cells in the absence of androgens as well as enhanced proliferation in response to very low levels of androgen [105]. Similarly, in intact non-castrate mice there were no differences in tumor growth between LNCaP xenografts and LNCaP xenografts stably expressing ARV567es; however in castrated mice, ARV567es tumors were larger compared to controls [105]. Furthermore, in support of this hypothesis, AR-V7 [101, 102, 116], AR-V1 [102], AR-V9, AR-V12 [103], and ARV567es [116] are all expressed at higher levels in CRPC specimens. These data suggest that AR variants are a valid therapeutic target in those men who progress while on ADT.

Subcellular Location of AR Variants

AR variants are structurally similar; however, as it has already been elaborated, may have distinct biological activities and functions. One obvious difference is the subcellular location of specific AR variants that may predict their activity. However, the cellular localization of AR variants may be cell-specific, dependent on the presence of full-length AR or androgen status. AR-V7 has been found to be localized to the nucleus in cultured cells under androgen-depleted conditions and constitutively active in driving the expression of genes including canonical androgen-responsive genes using reporter assays and microarray analysis [101, 102]. However, analysis of clinical specimens reveals a more complicated expression pattern. In benign prostate tissues, AR-V7 is mainly expressed in the basal and stromal cells with minimal staining in luminal epithelial cells. However, in malignant tissues, AR-V7 stained the majority of luminal cells in the cytoplasm and was nuclear in CRPC tissues [101]. This suggests additional variables are required for AR-V7 to localize to the nucleus.

AR8 does not contain a DBD and is therefore unlikely to function as a transcription factor. This lack of transcriptional activity was confirmed by testing several reporters driven by androgen-responsive promoters [107]. A further surprising observation from this study was that AR8 localized to the plasma membrane when overexpressed in AR-null COS-1 cells, whereas AR-V7 localized to the cytoplasm or the nucleus of COS-1 cells under the same conditions. Similar results were obtained when AR8 was overexpressed in LNCaP and CWR-R1 cells, suggesting that AR8 is preferentially associated with the plasma membrane [107]. This is possibly mediated through palmitoylation of two cysteine residues at codons 558 and 560 located within the unique COOH terminus amino acid sequence of AR8. Mutation of these putative palmitoylation sites led to loss of membrane localization of AR8 [107]. The primarily plasma membrane localization of AR8, coupled with the lack of a DBD, would suggest AR8 most likely functions through non-genomic mechanisms. This may be accomplished through EGFR, Src, full-length AR, and AR8 forming a dynamic signaling complex in response to EGF, and the level of AR8 may modulate kinetics of the assembly and dissociation of this complex, allowing sequential phosphorylation and subsequent nuclear translocation [107].

AR-V1 remains in the cytoplasm regardless of androgen status when overexpressed in AR-null COS-7 cells [106]. AR-V9 is also exclusively cytoplasmic [103]. AR-V12 [103] and ARV567es [105] localize to the nucleus regardless of the presence of ligand. Both of these AR variants retain the NLS in the AR exon 4-encoded hinge region, necessary for nuclear translocation [103, 105]. However, recent data suggest that AR variants such as AR-V7 which lack the canonical NLS, contain unique CTD sequences that reconstitute classical NLS activity and this is sufficient for nuclear localization and androgen-independent transcriptional activation of endogenous AR target genes [117].

AR Variant Interaction with Full-Length AR

AR variants are coexpressed with full-length AR in a number of cell lines, xenografts, and clinical samples. Analysis of AR-V7 and AR-V1 transcript expression by semi-quantitative PCR has shown that these AR variants are expressed at much lower levels (~0.1–2.5 %) relative to full-length AR in PCa metastases and several xenografts [106]. Therefore, the question whether AR variants interact with full-length AR is highly relevant. ARV567es is constitutively transcriptionally active and nuclear in the absence of androgens [105]. Importantly, when full-length AR and HA-tagged AV567es are overexpressed in AR-null M12 cells, both full-length AR and ARV567es are immunoprecipitated with an anti-HA antibody [105]. This suggests that ARV567es can functionally interact with full-length AR, perhaps resulting in increased stability of full-length AR as well as causing nuclear localization of full-length AR in the absence of androgens [105]. However, full-length AR/AR-V7 complexes have not been detected in 22Rv1 cells using standard co-immunoprecipitation techniques [96, 101]. Structural differences may account for the differences in interaction with full-length AR between ARV567es and AR-V7 due to AR-V7 lacking the hinge region encoded by AR exon 4 that is present in ARV567es. However, cell specificity cannot be ruled out. Furthermore, the tandem duplication of AR exon 3 in full-length AR in 22Rv1 cells which, encodes an additional zinc finger, has not been detected in other clinical specimens and may present a confounding factor by impeding interactions that could potentially be different than that observed with the wild-type full-length AR.

Overexpression of AR-V7 in LNCaP cells, which express full-length AR confers anchorage-independent growth in the absence of androgens [106]. This phenotype was reversed by treatment with the anti-androgen enzalutamide, which binds directly to the LBD, suggesting that AR-V7 is dependent on full-length AR [106], despite the observation that AR-V7 does not interact with full-length AR. More recently, it was observed that full-length AR is not required for AR-V7 transcriptional activity as demonstrated by silencing full-length AR with siRNA in LNCaP cells that overexpress AR-V7 [103]. Consistent with these data, androgen-independent growth of 22Rv1 cells is not dependent on full-length AR following knock down of its expression using two siRNAs targeting AR exon 4 and exon 6 encoding the LBD [118]. Taken together, these observations from two independent model systems suggest that at least some AR variants do not require full-length AR. However, further work to delineate the relationship between full-length AR and different AR variants is needed.

AR Variant Target Genes

Correlating androgen-regulated genes with genes expressed in CRPC has been a challenge. One potential explanation comes from the recent discovery that the gene expression program regulated by AR in androgen-dependent PCa cells is distinct

Table 13.2 Expression profiling of AR variant genes

Study	Tissue source	Experimental conditions	Profiling platform	GEO accession
[102]	LNCaP	Transfection of pcDNA-AR-V7 in 10 % CSS for 24 h ± 10 nM R1881 for 24 h	–	–
[101]	CWR-R1, 22Rv1	Transfection of shAR3-1 (knocks down AR-V7) or shARa (knocks down full-length AR)	Agilent 4x44K (G4112F)	GSE13919
[105]	LNCaP	Transfection of pcDNA-ARV567es or pcDNA-AR-FL in 10 % CSS for 24 h ± 1 nM DHT for 24 h	Agilent 4x44K	–
[120]	CRPC bone metastases ($n = 30$)	Comparison of CRPC bone metastases with AR-V567es and/or AR-V7 transcript levels in the upper quartile compared to other CRPC bone metastases	Illumina HumanHT-12V3.0	GSE29650
[121]	LNCaP	Transduction of lenti-AR-V7 in 10 % CSS for 24 h ± 1 nM R1881 for 24 h	Agilent 4x44K (G4112F)	GSE36549
	LNCaP	Transfection of pcDNA-AR-V7 or pcDNA-ARV567es in cells expressing stable clones of full-length AR positive and full-length AR negative cells in 10 % CSS		
	LNCaP95	Transfection of siRNA targeting AR-LBD or AR-DBD for 24 h ± 1 nM R1881 for 48 h		
	VCaP	Transfection of siRNA targeting AR-DBD or treatment with MDV3100 in the presence of 1 nM R1881		

from the one in androgen-independent cells [119]. Cell-specific and gene-specific transcription is thought to result from recruitment of different coregulatory proteins to the AR [119]. Therefore, it would be expected that truncated AR variants which lack regions of AR, would be devoid of some protein interfaces and/or have new interfaces as a result of the variable COOH terminus sequences. As a result, it is possible that specific AR variants may mediate distinct transcriptional programs in CRPC.

One hypothesis is that AR variants simply substitute for androgens and activate an identical repertoire of full-length AR target genes. Microarray analysis performed on RNA isolated from LNCaP cells ectopically expressing AR-V7 cDNA found that AR-V7 induced canonical androgen-responsive genes such as PSA, KLK2, NKX3.1, FKBP5, and TMPRSS2 in the absence of androgens (Table 13.2; [102]). Similarly, PSA, TMPRSS2 and FKBP5 induced by DHT treatment in cells

expressing full-length AR were also induced by ARV567es expression in the absence of androgens [105]. However, the authors of this study also identified a subset of genes regulated by ARV567es that are unique and not influenced by androgens in the context of full-length AR. Analysis of gene ontology (GO) terms enriched specifically in cells overexpressing ARV567es revealed that the GO molecular function "transcription factor activity" is significantly increased in the absence of androgen, signifying activation of other growth and survival pathways. Among the transcription factors upregulated by ARV567es that are known to induce a proliferative program of gene expression are STAT3 and JUN [105].

In order to identify AR-V7-regulated genes, Guo and colleagues employed shRNA constructs to selectively knock down AR-V7 or full-length AR in CWR-R1 and 22Rv1 cells and analyzed differential gene expression using microarray analysis. The differential expression of 188 genes was consistently detected in both cell lines when AR-V7 was knocked down, whereas the expression of 412 genes was altered in both cell lines when full-length AR was specifically inhibited. Among them, 71 genes are commonly regulated by both full-length AR and AR-V7 (e.g., IGFBP3, FKBP5). However, many well-characterized AR-regulated genes such as CLU, TMEPAI, PSA and CLDN4 were not affected by knockdown of AR-V7. Among the genes preferentially regulated by AR-V7, a number of genes such as MAP4K4, HOXB7, and ELK1 have been found to be upregulated in CRPC [101].

Recent data from gene expression profiling of CRPC bone metastases found 60 genes differentially expressed in bone metastases with high transcript expression of ARV567es and/or AR-V7 compared to other CRPC bone metastases. These genes included several gene transcripts known to be positively regulated by AR such as C-MYC, UBE2C, CDK1, CYCLINA2, and HSP27 but, interestingly, not some classical androgen-regulated genes such as PSA, TMPRSS2, and FKBP5 [120].

To dissect the transcriptional programs induced by AR variant-mediated signaling, Hu and colleagues [121] identified two core sets of AR variant and full-length AR-regulated genes, by assessing the transcriptional changes in LNCaP cells overexpressing AR-V7 in the presence and absence of full-length AR signaling by microarray and gene set enrichment analysis. Transient expression of exogenous AR-V7 in LNCaP cells induced expression of cell cycle genes under both androgen-depleted and androgen-stimulated conditions. Whereas, the top gene sets induced by ligand-dependent full-length AR were related to biosynthesis, metabolism, and secretion. The AR variant gene set was further characterized by overexpressing AR-V7 and ARV567es in stable clones of full-length AR positive and full-length AR negative LNCaP cells. Under these conditions, the AR variant gene set was induced by both AR-V7 and ARV567es, and the absence of full-length AR did not attenuate induction of the AR variant gene signature. This AR variant gene set was also upregulated in LNCaP95 cells (which endogenously express AR-V7 as well as full-length AR) and also VCaP cells, following siRNA targeted knockdown of the AR-LBD. These data suggest that the presence of full-length AR is not required for the induction of cell cycle genes by AR-V7 and ARV567es [121].

Therefore, it is likely that distinct gene expression profiles are mediated by AR variants versus full-length AR with some overlap. Clinically, these differences in

gene expression profiles may be relevant to biomarker discovery in order to identify those patients with tumors, which express AR variants that would not respond to traditional therapies.

Mechanism of AR Variant Production

The first mechanism to account for the production of naturally occurring truncated AR variants in the CWR22 models was calpain-mediated proteolysis [122, 123]. However, contradictory data also exists [124]. More recently an alternative mechanism for the generation of AR variants involving intragenic rearrangement of the AR locus within a region that is downstream from AR exon 2 and upstream to AR exon 4 has been reported in 22Rv1 cells [110]. Interestingly, long-term culture of androgen-dependent CWR22Pc cells, which exhibit neither AR variants nor intragenic rearrangement, in androgen-depleted media, leads to concurrent emergence of both [110]. These data suggest that AR intragenic rearrangements in CRPC may be associated with pathologic expression of AR variants during disease progression. Further intragenic rearrangements have been identified in the LuCaP 86.2 xenograft which expresses ARV567es. Using deletion-spanning PCR, an 8,579 bp deletion of AR exons 5, 6, and 7 in the LuCaP 86.2 xenograft was discovered, which provides a rational explanation for the synthesis of the truncated ARV567es variant [125]. The association of such genetic events with enhanced expression of AR variants in a large cohort of clinical specimens is needed to determine the clinical importance of this mechanism. Nevertheless, a genomic basis for the pathologic expression of AR variants may serve as a stable mechanism-based marker for resistance to ADT.

Implications for Treatment of CRPC

Currently, all hormonal therapies used in the clinic target the AR LBD directly with anti-androgens or indirectly by reducing circulating levels of androgens. Although this approach is initially effective, it is unclear why these approaches eventually fail. Compelling evidence from the last 5 years suggests that truncated AR variants lacking the LBD may be an important mechanism during disease progression to CRPC. AR variants retain the domain of the receptor that harbors the primary transcriptional transactivation function (i.e. AF-1); therefore there is a strong rationale for developing compounds that specifically bind the AR NTD to block activity. The first indication that the NTD is a viable target for in vivo intervention in CRPC was demonstrated with a decoy molecule encoding residues 1–558 of the AR NTD [126]. One particular challenge for the development of NTD antagonists is the fact that this region of the receptor is intrinsically disordered and therefore not amenable to crystallographic structural determination [127]. Recent studies have overcome this problem using cell-based screening protocols leading to the development of

EPI-001, a potent and specific AR NTD antagonist [128]. Preclinical studies have shown that EPI-001 treatment leads to cytoreduction of CRPC in xenografts dependent on AR for growth and survival without toxicity [128]. Analogues of EPI-001 are currently in development for clinical trials [129]. These findings presage a new era in targeting AR and are the first to demonstrate feasibility of targeting the AR NTD in vivo.

Conclusions and Future Directions

Remarkable advances in our understanding of the underlying mechanisms of the AR in disease progression to CRPC have been made over the last decade. There is now incontrovertible evidence that constitutively active AR variants represent another novel mechanism in disease progression, however much work is still needed to determine the function and mechanisms of individual variants. Differences in transcriptional activity of different AR variants have been reported and further studies are required to understand the molecular basis for these differences. It is possible that the different COOH terminus extensions of AR variants may provide important regulatory information, in addition to harboring the premature stop codon, which may influence DNA-binding activity or explain the cellular localizations of different AR variants. Furthermore, the mechanisms by which full-length AR can influence AR variant function (and vice versa) will also require further investigation. The gene expression program regulated by AR variants also remains to be fully elucidated, and this may provide a critical insight the mechanisms underlying AR variants in tumor progression to CRPC.

One of the most significant challenges will be the development and validation of a reliable, reproducible means of quantifying the levels and frequency of alternatively spliced AR variants relative to full-length AR in clinical specimens. This is of fundamental importance to establish the functional relevance of AR variants in disease progression and for the successful development and implementation of novel targeted strategies for patients with advanced stages of PCa, particularly those with progressive CRPC.

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Chapter 14
Biology and Clinical Relevance of Estrogen Receptors in Prostate Cancer

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Abstract This review explores the function of estrogen receptors (ERs) and their signaling pathways, and their involvement in the pathogenesis and management of prostate cancer (PCa). We pay special attention to (1) traditional estrogen receptors (ERα and ERβ) and the alternate estrogen receptor, G protein-coupled receptor 30 (GPR30); (2) therapeutic utility of estrogen and antiestrogens; (3) impact of genetic variants of ERs on prostate cancer risk; (4) epigenetic regulation of ERs; (5) downstream signaling of ERs; (6) diagnostic and prognostic value of these receptors; and (7) interaction between ERβ and aryl hydrocarbon receptor or androgen receptor. We also explore (a) the expression patterns of ERs and their spliced isoforms during normal development and the development and progression of PCa; (b) the divergent roles of the estrogen receptors as tumor suppressors and tumor promoters; (c) the possibility of developmental origin of the disease; and (d) the regulation of estrogen receptors via epigenetic modifications such as DNA methylation, histone modification, and microRNA processing. We also review the potential clinical application of various phytoestrogens (genistein, equol, dihydrogenistein, and daidzein), selective estrogen-receptor modulators (SERMs: tamoxifen, toremifene, and raloxifene), pure estrogen antagonists (e.g., fulvestrant), and transdermal estradiol delivered as preventive agents and first-line therapies or combinatory agents. We highlight the need for further studies of the role of ERs in epithelial–mesenchymal transition, prostate stem/progenitor cell function, and cross talk with other nuclear receptors and address the prospect of devising strategies for primordial prevention of PCa through improvements in the understanding of estrogen imprinting in early life.

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Introduction

Epidemiologic studies reveal the association between exposure to high levels of estrogens and the pathogenesis of prostate cancer (PCa). The difference in the levels of estrogens in members of different racial groups or from different geographical locations may explain the disparity in PCa risk around the world [1–3]. According to a 2012 report from American Cancer Society, the incidence rates in African–Americans are significantly higher than those in Caucasian Americans [4]. In the USA, African–Americans have higher levels of circulating estrogens and a higher incidence of PCa than do Caucasian Americans [5–8]. PCa is more common in North America and Europe than in Asia and South America [4]. A global study of levels of sex steroids in men 65 years or older found higher levels of estrogens, including estradiol (E2) and estrone (E1), in African–American men than in Asian men [9]. Another study found a correlation between the lower levels of circulating estrogen in Japanese men and a lower incidence of PCa when compared with Dutch men [10]. Moreover, higher levels of circulating E2 were significantly associated with the development of high-grade PCa [11]. Finally, a strong association was observed between increased levels of E1 and a higher risk of PCa in a cohort of men with a mean age of 73 years [12].

It has been known for some time that estrogen is involved in the development of the prostate gland and in the pathogenesis of prostatic disease [2, 11, 13–15]. Maternal E2, for instance, causes squamous metaplasia (SQM) within the developing prostatic epithelium in male offspring [16]. Treatment of dogs with an androgen plus estrogen has been used to experimentally induce benign prostatic hyperplasia (BPH) [16]. Moreover, PCa develops more rapidly in Noble rats given estrogens when under normal physiological levels of testosterone (T) [17–19]. This model is highly relevant to PCa development in humans because it mimics the alteration in the ratio of circulating estrogen to androgen that occurs as men age [20]. Treatment with T plus E2 also promotes the progression of PCa and its metastasis to distant sites such as lung and liver [21]. Furthermore, estrogens induce chronic inflammation in the prostate, a condition believed to increase PCa risk [18, 22, 23], although, these findings remain inconclusive [24].

In this review, we will place special emphasis on the following areas that address the biology and clinical relevance of estrogen receptor (ER)β in PCa: (1) traditional estrogen receptors (ERα and ERβ) versus the alternate estrogen receptor, G protein-coupled receptor 30 (GPR30); (2) the therapeutic utility of estrogen and antiestrogens; (3) genetic variants of ERs and epigenetic regulation of their expression; (4) downstream signaling mediators of these receptors; (5) diagnostic and prognostic values of these receptors; (6) the use of ERβ isoforms in management of PCa; and (7) the relationship between ERβ and aryl hydrocarbon receptor (AhR) or androgen receptor (AR).

ERs as Mediators of Estrogen Action

The estrogens have traditionally been believed to exert their effects primarily in the nucleus via the nuclear estrogen receptors ERα and ERβ [13, 16, 25, 26]. ERα, or NR3A1 (nuclear receptor subfamily 3, group A, member 1), is encoded by the *ESR1* gene, and ERβ, or NR3A2 (nuclear receptor subfamily 3, group A, member 2), is expressed from the *ESR2* gene. A membrane- or sarcoplasmic reticulum-associated G-protein-coupled estrogen receptor 1 (GPER), also known as the G protein-coupled receptor 30 (*GPR30*), is now recognized as a major alternative mediator of nonnuclear estrogen action [27].

ERα and ERβ

The functional domains of ERα and ERβ are arranged similarly. Because their DNA-binding domains are very similar (97 % amino acid homology), they can interact with specific *cis*-regulatory sequences such as the estrogen response elements (EREs) to transactivate common target genes [28–30]. Although both ERα and ERβ bind estrogens/antiestrogens at their ligand-binding domains, the domains share only about 56 % homology, leading to significantly disparate binding affinities for various ligands, including natural estrogens, phytoestrogens, and therapeutic estrogens [31–33].

The binding of an agonist to the ERα or ERβ elicits a conformational change in the receptor, resulting in a marked repositioning of helix 12 and the creation of new recruitment potentials of coactivators/corepressors in the AF-2 surface at the C terminus of these receptors [34]. These activated ERs are able to bind to EREs and serve as enhancers by ushering transcriptional factors and components of the transcriptional complex to the ERE sites, leading to the initiation of transcription of target genes. In contrast, antagonists alter the conformation of helix 12 in such a way that they block the binding of coactivators to the receptors, resulting in the inactivation of the receptor [35]. Although the receptor binding affinity (RBA) of ERα is greater for the natural estrogen, 17β-estradiol, than that of ERβ, the RBAs of ERβ for various xenoestrogens/phytoestrogens are greater than those of ERα. These include diethylstilbestrol (DES), nonylphenol, bisphenol A, isoflavones and their derivatives (genistein, dihydrogenistein, daidzein, equol), and apigenin [36, 37]. Moreover, the different N- and C-termini, which are responsible for interacting with co-regulators, account for the functional disparity between the two receptors [31, 38]. When ERα and ERβ bind to same ligand, they recruit different co-regulators/corepressors and dictate the use of different *cis*-regulatory elements either through direct binding or tethering to other transcriptional factors.

Consequently, ERα and ERβ regulate genes in different signaling pathways, significantly expanding their functional divergence [38–41]. Furthermore, genetic polymorphisms that exist in ERα and ERβ and the isoforms of ERβ add to the complexity of their actions (see below).

GPR30

GPR30, also referred to as G protein-coupled estrogen receptor 1 (*GPER*), is a member of the G-protein-coupled receptor (GPCR) superfamily. GPR30 is capable of mediating the rapid non-genomic responses induced by estrogen [27, 42, 43] in breast, endometrial, and ovarian cancer cells through the activation of Erk1/2 and cAMP pathways [43, 44]. During 1996–1998, four laboratories identified the receptor almost simultaneously in different cell types [45–48]. GPR30 consists of a seven-transmembrane-spanning, 375-amino acid receptor protein [49] localized in the plasma membrane [50–52] and/or in the endoplasmic reticulum [53–58], depending on either the cell type or cell conditions [42]. Three conserved cystine residues in the extracellular loops have been suggested to provide structural integrity by forming intramolecular disulfide bridges [46, 49]. A GPCR conserved D-R-Y amino acid triplet is believed to play a role in signal transduction. GPR30 contains a putative palmitoylation site that anchors the C terminus to the inside of the cell membrane. It also has four putative serine-, two threonine-, and one tyrosine-phosphorylation sites. Four alternate transcriptional splicing variants encode the same protein.

Estrogen binds to GPR30 with a high affinity [50, 59] to activate alternative estrogen signaling pathways. A specific synthetic agonist, GPR30-specific compound 1 (G-1), was identified in 2006 with the use of virtual and biomolecular screening. ICI 182,780 [59, 60], tamoxifen [60], and 4-hydroxytamoxifen (OHT) [61–63] also bind and function as GPR30 agonists. In addition, a variety of environmental estrogens can bind GPR30, including bisphenol A, zearalonone, genistein, nonylphenol, kepone, p,p'-DDT, o,p'-DDE, and 2,2',5' PCB-4-OH. However, their binding affinities are much lower than those of estrogen [64].

Estrogen, tamoxifen, ICI 182,780, and G-1 can bind GPR30 and then activate multiple cellular effectors such as mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), and phospholipase C (PLC), and activate calcium mobilization and cAMP production [42]. Most of these effects are mediated by *trans-activation* of epidermal growth factor receptor (EGFR).

GPR30 was recently found to localize to the cell nucleus upon binding to E2 and to elicit specific gene expression and cellular functions [65], thus blurring its traditional image as an estrogen receptor-mediating non-genomic estrogen action.

Role of Estrogen Receptors in the Natural History of Prostate Cancer Development and Progression

ERβ is the first ER to appear in the prostate during human fetal development (seventh week of gestation) and is the only ER subtype expressed in the epithelial and stromal cells during early ductal morphogenesis [66, 67]. ERβ is expressed throughout the ventral urogenital epithelium (UGE), ejaculatory ducts, müllerian ducts, and the entire stroma. At 8 weeks, ERβ staining is also observed in the prostatic buds. In contrast, ERα expression begins by week 15 of gestation, and at 19 weeks, strong ERα expression is seen in the luminal cells of the UGE, basal cells of the dorsal UGE, utricle, distal periurethral ducts, peripheral stroma, and posterior prostatic duct [67]. These findings suggest that ERβ, perhaps in concert with the AR, mediates the very early stage of fetal prostate development, followed by the action of ERα. Thus, selective ERβ-activating compounds may play an important role in prenatal estrogen imprinting of PCa risk. This supports the hypothesis that exposure to higher levels of E2 in utero leads to some of the differences in PCa risk among ethnic groups [68].

Differential expression patterns of the estrogen receptors are found in the epithelial and stromal compartments of the normal and malignant human prostate [69–77]. In the normal human prostate, the wild-type ERβ (ERβ1) is localized mainly in the basal epithelial compartment, where ERα is almost never found, whereas both ERα and ERβ are expressed in the stroma [72, 78]. Both ER subtypes are absent from the luminal epithelial compartment [72]. GPR30, on the other hand, is expressed primarily in the luminal epithelium [27] and is expressed in both normal and cancerous prostate.

Both ERα and ERβ have been proposed to play a role in the etiology of benign prostatic hyperplasia (BPH) [78]. Overall, ERα has a proliferative function in the prostate. In ERα knockout mice, reduced branching morphogenesis in the ventral and dorsal–lateral prostate, decreased fibroblast proliferation, and changes in stromal content were observed [79]. Moreover, levels of ERα were higher in the prostatic stroma of Hispanic and Asian men than in Caucasian and African American men, who are at a higher risk for prostate cancer [80]. ERβ, in contrast to ERα, has an antiproliferative function in the prostate. ERβ knockout mice develop prostatic hyperplasia in old age [81], and dogs exhibit an age-dependent decline in ERβ expression in the prostate [82]. In the human gland, the wild-type ERβ or ERβ1 was predominantly localized in the basal epithelial compartment of the prostate, and neoplastic transformation and progression in the prostate correlated with progressive loss of ERβ expression (see Fig. 14.1); yet, the receptor reappeared in PCa metastases in distant sites (lymph node and bone) [72], leading to the question of whether the receptor has a tumor-suppressor function in addition to a pro-metastatic potential in human PCa [83]. In addition, GPR30 was found to express in both cancer and adjacent normal tissue at comparable level as well as in bone and soft tissue

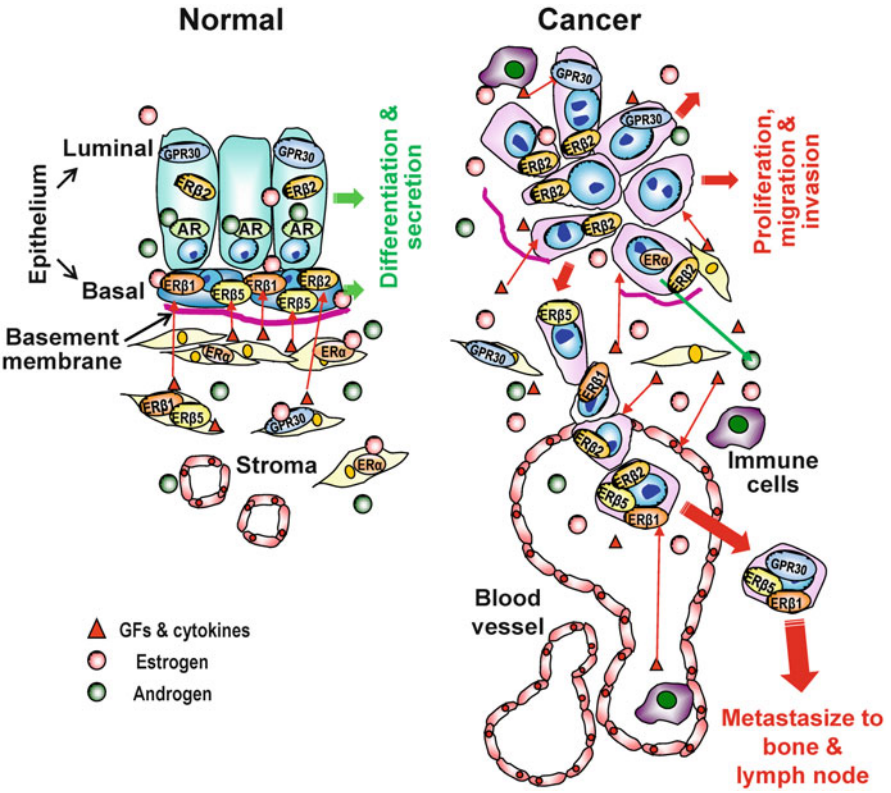

Fig. 14.1 *A schematic representation of the expression of various estrogen receptors in normal and malignant prostate tissues*. In the normal prostate, estrogen receptor (ER)β1 is localized principally in the basal epithelial cells, where ERα is almost never found. Both receptors are expressed in stromal cells. ERβ2 is expressed in both basal and luminal epithelial compartments as well as in the stroma. ERβ5 is strongly expressed in the cytoplasm of basal epithelial cells but not in the luminal cells, and weakly expressed in some stromal cells. Neoplastic transformation and progression in the prostate is associated with loss of ERβ1 expression, especially in high grade [4, 5] cancers. ERβ2 is the predominant ERβ isoform in the malignant cells, followed by some remaining expression of ERβ1; ERβ5 is the least common isoform. However, expression of nuclear ERβ2 and/or cytoplasmic ERβ5 in prostate cancer at prostatectomy predicts poor prognosis and early recurrence. GPR30 is found in both stromal and epithelial compartments of the normal gland. Its expression in cancer in the primary site diminishes in some cases but appears to increase in bone metastases. ERα in stromal cells are believed to produce cytokines and growth factors to influence the differentiation and tumorigenic potentials of the epithelial cells. In contrast, ERβ1 has been shown to have antiproliferative function while ERβ2 and ERβ5 promotes cancer cell migration and invasiveness. GPR30 activation appears to exert antitumorigenic action on prostate cancer cells

metastasis (Lam et al. unpublished). Further investigation is needed to uncover its function. Details of how genetic variation, spliced variants, and epigenetic regulation of estrogen receptors contribute to the pathogenesis and progression of PCa are discussed below.

Genetics: Polymorphism, Amplification, and Mutation

Genetic variation includes single nucleotide polymorphisms (SNPs), loss of heterozygosity, gene amplification, and mutation. To date, only SNPs of estrogen receptors have been reported to be associated with PCa. SNPs could modulate the expression and functionality of the estrogen receptors based on the location of the SNPs, ranging from residing in enhancer/promoter regions to the gene body. For example, SNPs at a promoter region may alter transcription efficiency and thereby modulate mRNA abundance. SNPs at other regions, such as in exons or at splice junctions, may result in altering protein stability, loss or gain of function, or formation of a nonfunctional decoy. Genetic variations of ERα and ERβ have been reported to be associated with PCa risk and severity of the disease.

Early studies on ERα polymorphisms in PCa focused mainly on two intronic variations, *ESR1 XbaI* [rs9340799] and *ESR1 PvuII* [rs2234693]. A study in a Caucasian cohort revealed that neither of these intronic variations alone was significantly different in the cancer and control groups [84]. When combined with a longer CAG repeat genotype in the AR, a homozygous *ESR1 XbaI* genotype (A/A) was more frequently detected in controls than in PCa patients [84]. Of interest is the finding of another study in mostly Caucasian men in which patients carrying G/G in *ESR1 XbaI* showed a shorter progression-free survival on chemotherapy than did other patients, and the presence of both mutant alleles (C in *ESR1 PvuII* and G in *ESR XbaI*) was related to the risk of developing castration-resistant PCa [85]. When other racial/ethnic groups were considered, the presence of the G allele in *ESR1 XbaI* genotype was associated only with an increase in PCa risk in African–American men—not in Hispanic or Caucasian populations [86]. But other studies suggested a significant association of a homozygous genotype of *ESR1 PvuII* (T/T) with higher risk in North Indian [87], Japanese [88], and British populations [89], whereas a study in an Iranian population suggested the opposite [90]. In contrast, studies with larger cohorts recruiting more than 400 cases and controls found no significant association of any SNPs in ERα, including the aforementioned loci, with PCa risk in a Caucasian [91], a non-Hispanic Caucasian [92], and two mixed populations [93, 94], particularly after adjustments for multiple comparisons. Moreover, there are a few studies investigating other SNPs, such as exon SNPs [95], population-specific tagging SNPs [96], or base repeats such as a TA or GGGA repeat, on ERα [97, 98]. Only two studies with base repeats [97, 98] and one coding SNP at exon 1 [rs17847075] [99], but not the other SNPs, showed an association with PCa risk. Overall, there is no single prominent SNP in ERα that strongly correlates with PCa risk across different racial/ethnic groups or populations.

Seven different studies have investigated the association of ERβ SNPs with PCa risk [90–92, 95–98]. The studied SNPs did not correlate with PCa risk with the following exceptions. A report (162 cases vs. 324 controls) in an Iranian population suggested an association of heterozygous AG at the *AluI* site [rs4986938] in the 3′ untranslated region of ERβ with increased risk [90]. A large Swedish case-control study also revealed a correlation between an SNP in the ERβ promoter region and

risk [100]. A pooled nested case-control study from seven cohorts observed that men carrying two copies of one of the variant haplotypes (TACC) had a 1.46-fold increased risk in multiple Caucasian populations [101].

For GPR30, multiple SNPs and one deletion polymorphism were identified to correlate with risk of other cancers or noncancer diseases [102–104], but information for PCa is not yet available.

Other genomic abnormalities, such as gene amplification or loss of heterozygosity for estrogen receptors, have not been found or clearly documented to be associated with PCa. However, a high frequency of allelic loss at 6q21–6q23, which is close to the ERα locus (6q23.1), was found in eight of ten cases of prostate carcinoma [105]. Similarly, gene amplification of an ERβ neighboring gene, HIF1A, was detected in PC3 cells. Its locus was mapped to 14q23, which is in close proximity to the ERβ locus (14q23.2) [106]. Nevertheless, such gene amplification was rarely found in PCa [106]. Genomic abnormality near the GPR30 locus has not been reported in any cases of PCa.

In conclusion, some of the SNPs in the various estrogen receptors, especially ERα, may associate with PCa in specific populations. However, whether they can be replicated in larger cohorts and multiracial/ethnic groups remains to be determined. The relevance of these genetic variations in PCa risk and severity needs to be investigated further.

Comparison of Isoforms or Spliced Variants with Wild-Type Receptors

Data from the post-genomics era suggest that one gene can encode multiple proteins. This could be achieved by alternative splicing events, including alternative usage of translated/untranslated exons or inclusion of introns. As a result, a premature protein sequence will be rearranged, leading to changes in subcellular localization via addition/removal of organelle-targeting signaling sequences or in functional alteration of the protein products through insertion/deletion of functional domains. In normal cells, this mechanism may be used to increase the diversity of the protein function. In contrast, cancer cells often hijack the splicing machinery to deregulate cell-cycle checkpoints and evade apoptosis. In this section, we discuss the role of spliced variants and isoforms of estrogen receptors in prostate carcinogenesis.

Bioinformatic analysis based on the National Center for Biotechnology Information (NCBI) database (GRCh37/hg19) found that *ESR1*, the official gene symbol for ERα, makes up eight coding exons [107]. However, four 5′-translated or untranslated exon splice variants have been validated and registered for ERα (accession number NM_001122742, NM_001188741, NM_001122740, NM_000125). This suggests that ERα may potentially utilize four different promoters, depending on various tissue contexts. In addition, more than a dozen splice variants have been sequenced and submitted to the NCBI genome database [107]. Exon skipping of the

body of the ERα gene is one of the common events for most splice variants. Several ERα splice variants with skipping exons have been amplified from various PCa cell lines [108]. Since skipping one or two exons is likely to cause in-frame or out-frame deletions, such ERα variants may lose one or multiple functional domains, possibly forming dominant-negative proteins, i.e., proteins that compete for the same ligand without initiating the downstream functions. Surprisingly, no transcriptional or dominant-negative activity was demonstrated in vitro [108]. In a pilot study of a small mixed population, one of the ERα splice variants missing exon 5 (ERαΔ5) was expressed greater in tumor-adjacent prostate samples than in benign samples [109]. More in-depth studies are required to elucidate the roles of ERα variants during prostate carcinogenesis.

ERβ, or *ESR2*, however, shows a different splice-variant profile. ERβ is composed of eight translated exons and at least one 5′ untranslated exon. Exon skipping in the gene body (exons 1–8) of the ERβ gene is not very common. Instead, alternative usage of 5′ untranslated (also termed exon 0) and 3′ translated exons (exon 8) is often found in ERβ transcripts. There are at least two 5′ untranslated exons, namely *0N* and *0K*, spliced into exon 1 and found in multiple tissues [110–112], including the prostate [113, 114], suggesting that at least two different promoters (promoter *0N* and *0K*) regulate *ESR2* transcription. Their promoter activity has been determined in PCa cells [114]. Although promoter *0N* showed significantly higher transcription activity than promoter *0K*, both exon *0N*- and *0K*-initiated transcripts are readily detected in PCa specimens by quantitative PCR and in situ hybridization (Unpublished work). Intriguingly, eight untranslated exons, termed *0X*1 to *0X*8, have recently been found to be randomly inserted between exon *0K* and exon 1 of the ERβ transcripts [115]. The function of exon *0X*s is not clear. Sequence analysis of all exon 0X-associated transcripts predicts the presence of several upstream open reading frames (uORFs) with start and stop codon pairs preceding the protein coding sequence of ERβ (Unpublished work). The existence of uORF in the 5′ untranslated region of a transcript is known to inhibit protein translation [116]. This uORFs can serve as another mechanistic layer in regulating ERβ expression in addition to traditional transcriptional control. Regarding the 3′ end of ERβ, five different coding exons 8 were amplified and sequenced from various tissue sources [117, 118], as well as from the prostate [75, 119]. Since each of the 3′ translated exons has a unique sequence and contains a specific stop codon, alternative use of these exons will produce different ERβ protein sequences with unique C-termini encoding AF2 domains of different length, forming ERβ isoforms 1–5. The *ERβ* sequence originally discovered in human testis is now referred to as *ERβ1* [31]. All other isoforms were named ERβ2 to ERβ5 according to Moore et al. [117]. Transcript expression of ERβ isoforms has been evaluated in PCa cell lines [119] and PCa tissues (Unpublished work). In general, ERβ1, 2, and 5 show moderate to high levels of expression in cell lines and PCa specimens on RT-PCR. Full-length ERβ isoforms have been cloned [118, 119], and their functions have been investigated in a classical ERE-mediated transactivation assay [119]. ERβ1 is the only isoform that responds to ligands and transactivates reporter expression, whereas the other isoforms (ERβ2, 4, and 5) do not respond to common ERβ ligands [119]. This lack of

response could be due to the absence of or low ligand affinity and a lack of functional helix 12 formed from the truncated AF2 domain [119]. As a result, all ERβ isoforms except ERβ1 are incapable of recruiting coactivators such as SRC-1 to initiate gene transactivation [119]. Interestingly, ERβ2, 4, and 5 can form heterodimers with ERβ1 and augment ERβ-mediated *TFF1* (also called pS2) gene expression only when E2, but not genistein, is used as a ligand [119].

Immunohistochemical analyses using an isoform-specific antibody revealed a progressive loss of nuclear staining of ERβ1 from high-grade prostatic intraepithelial neoplasia (PIN) to Gleason Grade 5 cancer foci but its restoration in PCa cells that metastasized to lymph node and bone [113]. This suggests that ERβ1 may have a tumor-suppressor function in localized cancer but becomes cancer promoting when PCa cells leave the primary site. In a cohort of 144 patients with long-term follow-up, nuclear ERβ1 was not associated with either recurrence- or metastasis-free survival [75]. In agreement, the ectopic expression of ERβ1 in PC3 cells also was associated with a significant reduction in cellular migration and invasiveness [75]. In the same study, the presence of ERβ2 in the nucleus correlated with rising prostate-specific antigen, postoperative metastasis, and shorter recurrence- and metastasis-free survival [75]. This association is in concordance with in vitro data indicating that ERβ2 promoted invasiveness [75]. In fact, an earlier study with 48 cases also concluded that ERβ2 is a poor prognostic marker for cancer-specific survival [120]. The cancer-invasive property of ERβ2 is further supported by two recent studies: (1) ERβ2 mediated the expression of gastrin-releasing peptide (GRP) receptor and, in turn, stimulated cellular proliferation and invasiveness of androgen-independent PCa growth [121] and (2) ERβ2 upregulated the expression of bone-metastasis genes in PC3 cells [122]. In contrast, not much is known about the function of ERβ5 in prostate carcinogenesis except for our finding that cytoplasmic staining of ERβ5 was associated with a shorter time of metastasis-free survival and that ectopic expression of ERβ5 promoted cellular migration and invasion [75]. Whether ERβ2 and ERβ5 share common signaling pathways that exacerbate PCa invasiveness requires further investigation (see Fig. 14.1).

Epigenetic Regulation of the Expression of Estrogen Receptor

The expression of estrogen receptors is governed by various epigenetic regulatory mechanisms. Epigenetic changes do not involve alteration of primary DNA sequences and are mitotically heritable and potentially reversible. Cancer cells often reprogram oncogene and tumor-suppressive gene networks through epigenetic events [123]. One of the most well-defined epigenetic events is DNA methylation. It involves the addition of a methyl group to a 5′ cytosine neighboring with a guanine (CpG) to form a methylated CpG (mCpG). A high density of multiple mCpGs in the regulatory regions of a gene, often referred to as a CpG island, recruits DNA-methylation silencing complexes containing methyl CpG-binding proteins (MeCP2,

MBD1-4) and histone deacetylases (HDAC) that block RNA polymerase II from binding to the transcription start site, leading to gene silencing [124]. Another well-known epigenetic mechanism involves posttranslational modifications of histones such as acetylation and methylation at multiple lysine residues in the N-terminal end of histone 3 or 4. For instance, acetylation at lysine 4 of histone 3 is frequently found associated with promoters of active genes [125] while tri-methylation at lysine 9 of histone 4 is often located to promoter regions of repressed genes [126, 127]. Additional epigenetic changes are mediated by microRNA (miRNA) action at the posttranscriptional level. A miRNA forms complete or incomplete duplexes with one or more target sequences in the 3′ untranslated region of its target mRNAs via full or partial sequence complementarity, respectively [128]. This in turn recruits RNA-induced silencing complex (RISC) and initiates transcript degradation or inhibition of protein translation [128].

DNA Methylation

ERα transcript and protein were often undetectable in malignant primary prostate epithelial cells [129]. Dahiya and his associates showed a progressive increase in methylation of ERα from 60 % in the BPH samples to 95 % in the high Gleason grade tumors [130]. Another report published by the same group discovered that three of the ERα promoters were flanked by a cluster of CpGs (CpG islands) and revealed that two of the promoters were hypermethylated in 36 of 38 cases of cancer but observed no methylation in all normal tissues [131]. The gene regulatory function of these CpG islands was validated using PCa cell lines treated with a demethylating reagent, 5-aza-2′-deoxycytidine, to restore ERα expression [131]. Other studies have also reported this preferential loss of ERα together with augmentation of its promoter methylation in cancer tissues [132, 133]. Interestingly, when patients were stratified by age, those 70 years or older showed a higher degree of ERα promoter methylation than patients 60 years or younger, but this association was not independent of Gleason score [134].

ERβ was barely detectable in PCa and malignant primary prostate epithelial cells [113, 129, 135]. This loss of receptor expression was shown to be regulated by progressive hypermethylation of the ERβ promoter during prostate carcinogenesis in the primary site [113, 131, 135]. Using immunostaining-guided laser-capture microdissection, we analyzed 41 CpG sites of the ERβ *0N* promoter in 56 clinical samples, including BPH, adjacent normal, PCa of different grades, lymph nodes, and bone metastases [113] and observed an inverse correlation between the degree of CpG methylation in the promoter and loss of immunopositivity of the receptor in the various tissue types [113]. In contrast, we observed promoter hypomethylation and “reappearance” of ERβ expression in bone and lymph node metastases [113]. This study also identified three CpG hypersensitive hotspots for methylation changes, two located in the exon region and one in the promoter region [113].

Hypermethylation of the hotspot in the promoter region effectively silenced ERβ expression in vitro and in vivo [113]. A follow-up investigation based on this study identified AP-2 as the key transcription factor interacting with this methylation hotspot responsible for regulating ERβ transcription [114].

GPR30 may potentially be regulated by DNA methylation, as a CpG island with 29 CpG sites is predicted in the first untranslated exon of two splice variants and the 5′ regulatory region of another GPR30 splice variant. Moreover, it is important to note that the coding sequence of GPR30 is very rich in CpG, with 106 CpG sites. Whether a methylation change in these CpG sites can affect GPR30 transcription needs further exploration, as no information is yet available.

Histone Modifications

ERα can be silenced by histone modification in other cancers [136–138]. However, information regarding this mode of epigenetic regulation of ERα in PCa is limited to one report showing restoration of ERα expression with the treatment of LNCaP cells with trichostatin A (TSA), an HDAC inhibitor [139].

ERβ expression in PCa cells was restored with treatment with HDAC inhibitor [139, 140]. It is noteworthy that different PCa cell lines show different sensitivities to HDAC and DMNT inhibitor treatment [139]. In both DU145 and LNCaP cells, ERβ expression was sensitive to HDAC treatment, but PC3 cells were less sensitive. The latter cell line responded to treatment with DNMT inhibitor better than to treatment with HDAC inhibitor with regard to restoration of gene expression [139]. Epigenetic regulation of GPR30 through histone modification is virtually unexplored.

MicroRNA

At least eight miRNAs (miR-22, miR-206, miR-221, miR-222, miR-18a, miR-18b, miR-193b, and miR-302c) have been determined to regulate ERα, primarily in studies of breast cancer [141]. Although the relationship between ERα expression and miRNAs in PCa has not been fully established, several of these miRNAs were found to be associated with the cancer. MiR-22 may have an association with PCa, but the results from two studies were contradictory and inconclusive [142, 143]. MiR-206 expression did not strongly correlate with the progression of PCa [144]. MiR-193b was found to be a tumor suppressor [145], possibly through downregulation of urokinase plasminogen activator in PCa cell models [146].

For ERβ, only one miRNA, miR-92, was shown to specifically target the receptor in breast cancer [147], and the function of this miRNA in PCa remains unknown. No experimental data are yet available for GPR30 and miRNAs in any cancer type.

Tumor-Suppressor and Tumor-Promotion Action of Estrogen Receptors

Accumulated evidence suggests that ERα promotes prostate carcinogenesis. ERα plays a significant role in E2-induced squamous metaplasia (SQM) in an ERα knockout (alpha-ERKO) mouse [148]. Tissue recombination experiments suggested that ERα in both epithelial and stromal compartments are important for the evolution of SQM [148]. But in studies of a transgenic mouse in which epithelial ERα, but not stromal ERα, was knocked out, SQM formation was greatly reduced, suggesting that perhaps epithelial ERα plays a more important role [149]. In human prostate, prostatic SQM is frequently observed at the end of gestation and is considered to be a normal physiological process [150, 151]. Nevertheless, ERα was identified as a major player in neonatal estrogenization-mediated prostatic dysplasia in alpha-ERKO mice [152] and in a chronic T-plus-E2-induced PIN rodent model [153]. SERMs were used to target ERα in animal models. Trioxifene inhibited metastasis and extended survival in the PAIII rat model [154], whereas toremifene effectively reduced cancer incidence by 65 % in the transgenic adenocarcinoma (TRAMP) mice, with extended survival [155]. Prolonged treatment with T plus ERA-45, an ERα-specific agonist, caused precancerous PIN lesions in rat prostate [156]. In humans, since ERα was readily detected in HGPIN epithelium [157], a clinical trial of treatment and prevention of HGPIN with toremifene was conducted [158]. Cancer incidence was significantly reduced in the group treated with 20 mg of toremifine as compared with the placebo group [158]. Overall, these findings support the concept that ERα has tumor-promoting properties during prostate carcinogenesis. One proposed mechanism of this action is that ERα augments epithelial cell invasiveness by increasing the level of stromal secretion of metalloproteinase 2, which is under ERα-mediated upregulation of TGFβ signaling [159].

A majority of studies suggest that ERβ is a tumor suppressor during an early stage of PCa development. ERβ knockout causes prostatic hyperplasia and dysplasia in transgenic mice [81, 160], implicating an antiproliferative activity of ERβ [161]. But studies in other ERβ knockout mice models did not confirm this observation [162–164], perhaps because of a drift in the mouse genetic background or in differences in the strategies of developing the various ERβKO mice. In humans, ERβ expression in epithelial cells is lost as cancer develops and advances to higher grades in the prostate [70, 72, 113, 129, 165]. An inverse correlation between ERβ and epithelial–mesenchymal transition (EMT) markers was reported in higher-grade PCa [166]. The latter finding corroborates those from animal studies showing a protective role of ERβ during early stages of prostate carcinogenesis. However, ERβ was also found in lymph node and bone metastases [69, 113, 167], and its role in cancer metastasis remains unclear. Two studies suggested that ERβ may promote the spreading of PCa, as ERβ immunopositivity correlates with high-grade PCa and decreased relapse-free survival time [70, 168]. Immunopositive signals of ERβ in some of these association studies could be compromised by the use of pan ERβ antibodies, which are unable to differentiate ERβ1 from its isoforms, because

expression of ERβ2 and ERβ5 in PCa was shown to be associated with poor prognosis and increased PCa cell migration and invasion [75]. In cell-based models, ERβ1 not only suppressed cell proliferation [169] via cell-cycle arrest [170] but reduced cell migration [75, 171] and invasion [75, 169], partially through tight regulation of the EMT pathway [166] and by triggering apoptosis [169] via TNFα signaling [172] and anoikis through tethering with KLF5 [173]. In conclusion, both observational and cell-based studies support a tumor-suppressive activity of ERβ1 during early carcinogenesis but the possible promotion by ERβ1 of metastasis once the PCa cells leave the primary site. The roles of the isoforms remain elusive, but they likely promote progression.

Studies on GPR30 in PCa are limited. The consequences of GPR30 signaling pathways often lead to tumor promotion. A number of in vitro studies have demonstrated that activation of GPR30 stimulates cell proliferation in breast cancer, endometrial cancer, ovarian cancer, and thyroid cancer through the EGFR-MAPK signaling pathway. In contrast, activation of GPR30 by estrogen and G-1 inhibited growth of PCa cells in vitro by activating Erk1/2 and upregulating prolonged p21/Waf1-mediated G2-M arrest. Moreover, G-1 treatments attenuated the growth of human PCa xenografts in nude mice [174]. This suggests that a specific GPR30 ligand such as G-1 has therapeutic potential for PCa. Moreover, quantitative RT-PCR analysis of seven pairs of clinical PCa samples and their corresponding adjacent normal tissues showed that a trend of slight reduction in GPR30 transcript levels was present in cancer specimens, whereas the majority of the samples expressed GPR30 [175]. Similar results were found in expression profiling in Oncomine [175]. Further study of GPR30 protein expression in a large cohort of samples is clearly warranted (see Fig. 14.1).

Estrogen Receptors as Therapeutic Targets

Diethylstilbestrol

ERs, which play important roles in the pathogenesis and progression of PCa, have been used as therapeutic targets of hormonal therapy. Diethylstilbestrol (DES), a xenoestrogen, was the first effective hormonal drug therapy for PCa [176]. It was used as the standard initial treatment for metastatic PCa starting in 1941 [176, 177]. However, it lost its popularity because of cardiovascular toxicity and other adverse side effects, e.g., gynecomastia, nausea, vomiting, and thromboembolism, and was gradually replaced by other hormonal therapies, such as the gonadotropin-releasing hormone analogue leuprolide [76, 177–179]. Recently, the use of low-dose DES for the treatment of castration-resistant PCa has been tested and found to have a palliative benefit [180, 181]. It is considered as an alternative option in the pre- or post-chemotherapy setting [180, 182].

Tamoxifen and SERMS

Selective ER modulators (SERMs) are estrogenic compounds acting as agonists or antagonists of different ER subtypes in a cell-/tissue-specific manner [183]. Tamoxifen, toremifene, and raloxifene are different generations of SERMs used for the treatment of different cancers [184–189]. However, clinical trials, mostly phase 1 or 2, have demonstrated that these agents have limited efficacy in the treatment of PCa. The lack of efficacy of these therapies eventually limited their use as alternatives to DES. Part of the reason for their inefficacy as PCa therapies could be due to the fact that they were originally designed to target ERα in breast cancer [183]. Recent studies have shown that the combined use of SERMs with other therapies can reduce the incidence of side effects. Toremifene together with androgen-deprivation therapy significantly increased the bone-mineral density and reduced the fracture risk of PCa patients [189–191]. Tamoxifen effectively prevented gynecomastia in patients receiving bicalutamide monotherapy [192–194].

Preliminary clinical studies with the ERα antagonist toremifene have identified ERα as a promising target for prevention of PCa. The partial loss of the ERβ in HGPIN indicates that ERβ1 acts as a tumor suppressor.

Transdermal E2

The use of estrogen patches, a recent development in the treatment of PCa, has grown in popularity [195–197], and randomized, controlled, multicenter phase 2 trials as first-line hormonal therapy in patients with locally advanced or metastatic PCa are now in progress [196]. Preliminary results indicate that the levels of testosterone and PSA response are similar to those of castration [197]. The advantages of transdermal patches include reduction in cardiovascular toxicity, maintenance of adequate hormonal levels for a convenient time period [198], the alleviation of hot flashes, and improved bone density after endocrine treatment of PCa [199, 200]. A separate trial of 22 patients with refractory aggressive tumors after androgen ablation therapy and chemotherapy as demonstrated by a median PSA of 170 ng/ml (range, 14–5,030 ng/ml) is now complete [201]. Nine patients had a decrease in PSA and two had a PSA response defined as a 50 % decline in PSA. Therapy was well tolerated, and no thrombotic events were observed. Transdermal estrogen patches thus offer a promising outcome for patients with PCa.

Fulvestrant

Fulvestrant (ICI 182,780) is a promising therapeutic drug for PCa in preclinical models. In DU145 cells, fulvestrant suppresses cancer cell growth, modulates NFκB

[202, 203] and KLF5 signaling pathways, and triggers anoikis in mouse xenografts via tethering with ERβ [173]. Fulvestrant can activate the expression of hsa-miR-765 via ERβ in DU145 cells; suppress a known oncogene, HMGA1 [204, 205], as one of the novel targets of hsa-miR-765; and reduce cellular proliferation, migration, and invasion (Unpublished work). Fulvestrant can reduce the growth of LNCaP cells by suppressing the activity of the mutated-877 androgen receptor (AR) [206] and AR protein expression [207]. Fulvestrant can also regulate stromal cell functions. Treatment of primary prostate stromal cells with fulvestrant reduces cell viability [208]. As a pure antagonist for ERs, fulvestrant can antagonize E2-mediated cell proliferation and E2-induced MMP2 expression [159, 209].

Fulvestrant has also been tested in several rodent models where it reduces the Ki-67 labeling index and IGF-I receptor levels in rat prostate epithelium [210]. In an E2-induced PCa rodent model [211, 212], fulvestrant blocked E2-mediated epithelial cell proliferation and dysplasia by reversing E2-regulated genes, as well as by inducing fulvestrant-specific gene signature [213–215]. In the Wistar rat model, fulvestrant downregulated AR expression in ventral prostate and reduced ERK1/2 phosphorylation and prostatic epithelial cell proliferation [216].

One phase II clinical trial of fulvestrant has been reported [217]. Twenty patients with castration-resistant PCa (CRPC) were treated with fulvestrant based on the loading-dose regimen (500 mg on day 0 and then 250 mg on day 14, day 28, and monthly thereafter). No favorable clinical or PSA response was observed, as 80 % of the patients had metastasis and 20 % showed rising PSA levels after 6 months of treatment. However, fulvestrant did not cause severe toxicity [217]. Thus, by changing the loading dose to 500 mg every 14 days for the first month and 250 mg monthly thereafter, fulvestrant effectively reduced PSA levels by 40–99 % within 0.27–2.67 months in six of the seven highly pretreated CRPC patients without any obvious toxicity [218]. Importantly, PSA levels in these patients bounced back soon after the treatment was reduced to 250 mg a month [218], indicating that CRPC is highly sensitive to fulvestrant dosage. In the future, a better loading-dose regimen with optimal fulvestrant dosage may be used as a second line of treatment for patients with CRPC. Another small-scale clinical trial was conducted with a single 250-mg dose of fulvestrant 28 days before prostatectomy (Unpublished work). The tumor-suppressive miRNA, hsa-miR-765, was significantly upregulated, and its downstream oncogene target, HMGA1, was significantly downregulated in the fulvestrant-treated prostate specimens. This suggests the possibility of using fulvestrant in neoadjuvant therapy for prevention of cancer progression and recurrence.

Phytoestrogens: Genistein, and Equol

Phytoestrogens are an important class of estrogen-based chemopreventive agents. In Asian epidemiologic studies, health benefits, including reduced incidence of

breast cancer and PCa, are attributed to soy food and isoflavone consumption [219–223]. The most common phytoestrogens with estrogenic or antiestrogenic activities are soy isoflavones such as genistein, equol, and daidzein, all abundant in soy beans and its products [224–227]. However, cohort studies [228–235] indicate that the value of the protective or therapeutic effects of phytoestrogens is still controversial. Nevertheless, a number of phase 1 clinical trials evaluating the use of phytoestrogen supplementation in patients with PCa have generally seen beneficial effects without any toxicity [236–240]. For example, a placebo-controlled, block-randomized double-blind phase 2 study [241] examining the effect of a daily dose of synthetic genistein on serum and tissue biomarkers in patients with localized PCa indicated that a dose of genistein, which can be easily obtained from a diet rich in soy, reduced the level of serum PSA in patients with localized PCa by 7.8 %, without any effects on hormones.

2-Methoxyestradiol

The enzyme catechol-*O*-methyltransferase (COMT) is responsible for the inactivation and removal of genotoxic estrogen-derived intermediates such as 2- and 4-hydroxyl catecholestrogens and their quinone/semiquinone intermediates that act as chemical carcinogens [242] via the formation of DNA adducts. Increased expression or activity of COMT protects against estrogen-induced cancer by mediating the conversion of catechol estrogens into methoxyestrogens that have potent apoptotic activity against rapidly growing PCa cells [243]. This has prompted the testing of combinatory therapies involving methoxyestrogens and other standard therapies, such as hormone deprivation [244], docetaxel [245], and eugenol [246], against PCa growth in model systems.

Combinatorial Therapies

New therapies for PCa are constantly being sought. For example, a novel nonsteroidal selective estrogen receptor-α agonist (3-fluoro-*N*-(4-fluorophenyl)-4-hydroxy-*N*-(4-hydroxyphenyl) benzamide named as GTx-758) recently was designed as first-line therapy for advanced PCa. In animal models, GTx-758 induced medical castration and minimized many of the estrogen deficiency-related side effects of androgen deprivation therapies. Orally administered GTx-758 reversibly suppressed testosterone to castration levels and subsequently reduced prostate volume and levels of circulating prostate-specific antigen in relevant preclinical models (aged rats) without inducing hot flashes, bone loss, thrombophilia, and hypercoagulation or increasing fat mass [247].

Emerging Areas/Future Direction

Prostate Stem Cells Vs. PCa Stem Cells

Prostate progenitor cells are believed to reside mostly in the basal layer [248, 249]. Those cells are able to undergo asymmetric proliferation, self-renewal/regeneration, and differentiation and to become luminal epithelial cells and neuroendocrine cells. One of the classical experiments to demonstrate the existence of prostate progenitor/stem cells is castration-regeneration experiments in rodent models. After depletion of androgen, prostate acini become atrophic; most of the luminal epithelial cells regress or die off due to apoptosis [250]; and only the epithelial cells, which are androgen-independent and castration-resistant, can survive [249]. This is consistent to other observations that prostate cells with progenitor properties do not express androgen receptor [251–253]. Treatment of the castrated animals with androgen restores normal prostatic epithelium with three lineages of cell type [249], suggesting that the castration-resistant cells are capable of regenerating normal prostate acini and demonstrating the tissue-regeneration property of the castration-resistant cells. Also, these cells can survive repeated castrations [249], implying that the resistant cells are able to undergo asymmetric proliferation to maintain the progenitor population. Recent studies suggest that some of the luminal epithelial cells [254] may retain progenitor functions. A PCa stem cell hypothesis has recently been proposed in response to the experimental observation that a small piece of human PCa tissue transplanted into immunodeficient mice can propagate and maintain cellular heterogeneity of primary prostate tumors [255, 256]. A similar concept has also been tested using primary PCa cell lines to reconstitute the original tumor in tissue recombination experiments [253]. However, it is still unclear whether PCa stem cells originate from normal prostate stem/progenitor cells or are derived from different lineages via dedifferentiation.

The role of estrogen receptors in prostate stem/progenitor cells has not been fully explored. Nevertheless, neonatal exposure of the prostate to estrogen was found to give rise to PIN lesions and to be mediated through ERα, but not ERβ, in transgenic mice models [152]. This suggests that estrogen may reprogram prostate stem/progenitor cells via ERα during an early stage of prostate development. E2 also was found to increase the number and size of prostaspheres, where ERα, ERβ, and GPR30 are readily detected [257, 258]. But the estrogen receptors involved have not yet been defined. The role of ERβ in prostate stem/progenitor cells has begun to be more fully appreciated. The use of an ERβ-specific agonist to target stem cell-related prostate diseases has been attempted, as administration of this ligand can deplete the remaining ERβ-positive basal epithelial cells or stem/progenitor cells after castration [259]. There may be a promising future for the development of strategies to prevent prostate diseases if we can better understand the role of estrogen receptors in prostatic stem/progenitor cells.

Early Origin of PCa Through Estrogen-Receptor Action

Although most PCa develops in men 65 years and older [4], PCa risk can be determined by early life exposure to estrogens through a mechanism known as "estrogen imprinting" [260]. Different animal studies have shown that perinatal or neonatal exposure of rodents to estrogens or compounds with estrogenic activity leads to inflammation, stromal hypertrophy, elevated proliferation, and changes in the levels of AR and ERs in the adult prostatic gland [261–267]. Moreover, when the prostate is again exposed to estrogen as a second hit during adult life, PIN occurs in the affected gland [268]. Studies in an animal model indicated that epigenetic reprogramming during the early stage of prostatic development may be one of the mechanisms leading to estrogen imprinting [268]. Specifically, the promoter regions of *nucleosome binding protein 1* (*Nsbp1*), *phosphodiesterase type IV* (*Pde4d4*), and *hippocalcin-like protein 1* (*Hpcal1*) were aberrantly methylated in adulthood when the neonates were exposed to estradiol or bisphenol A during the postnatal period. A host of enzymes/proteins responsible for DNA methylation were also permanently affected by this early life exposure. Epidemiologic findings support the hypothesis that elevated exposure to estrogen in utero correlates with increased PCa risk. For example, higher levels of circulating E2 were found in pregnant African–American women, whose children have a higher chance of developing PCa than do the children of Caucasians-American women [5–8, 269]. Infants with high birth weight and jaundice, which are the indicators of a high E2 level during pregnancy, are associated with increased PCa risk. In contrast, preeclampsia in pregnancy, which is an indicator of low E2 levels, is related to a lower incidence of PCa [250, 270]. Since PCa may be a fetus-based disease, early life exposure to other sources of estrogen should not be ignored. Significant exposure of fetuses to bisphenol A (BPA) through the maternal use of BPA-containing products has been well documented [271–275]. To conclude, if PCa risk can be determined in early life, primordial cancer-prevention strategies could be devised to significantly reduce the suffering and healthcare burden from PCa.

Microenvironment: EMT/MET and Stroma Factors/Field Effects

Experiments with ERα and ERβ null mice have demonstrated that ERα is essential in the induction of prostatic squamous metaplasia. The respective roles of epithelial and stromal ERα in this response were determined by constructing tissue recombinants with prostatic epithelia and stroma from wild-type and ER knockout mice. The development of full and uniform epithelial squamous metaplasia required the presence of ERα in both the epithelium and stroma [148, 276], indicating the importance of cell–cell interactions in the mediation of estrogen action in the prostate via ERα.

Unlike ERα, ERβ1 exerts an antiproliferative effect on the prostate epithelia [39, 172] and inhibits epithelial–mesenchymal transition (EMT) [166]. The VEGF-A receptor neuropilin-1 drives EMT by promoting Snail1 nuclear localization. ERβ destabilizes hypoxia-inducible factor 1α (HIF-1α) and induces the transcriptional repression of VEGF-A and thus the inhibition of VEGF-mediated Snail1 nuclear localization [166]. Also, loss of ERβ promotes EMT, while stimuli (TGF-β and hypoxia) that induce EMT diminish ERβ expression. Moreover, the co-expression of ERβ with endothelial nitric oxide synthase (eNOS), Hif-1α, or HIF-2α is found in aggressive cancers, suggesting that an estrogen-mediated NO-enriched environment may influence the aggressive phenotype of PCa significantly [168]. ERβ and the eNOS complex has also been found to repress transcription of prognostic genes that are downregulated in PCa, such as the glutathione transferase gene GSTP1 [277]. Thus, differential binding of the receptor to different proteins and cross talk with EMT-signaling cascades introduce additional divergence in ERβ action. In addition to the antiproliferative role of ERβ1, the two other ERβ isoforms ERβ2 and ERβ5 have recently been demonstrated to promote metastasis [75]. Thus, ERβ appears to play various roles in human prostate carcinogenesis through differential expression of the various spliced variants and possibly through alternative promoter utilization. The mechanism underlying this ERβ2- and ERβ5-specific effect needs to be investigated further.

Notably, proteins involved in EMT can serve as biomarkers for more aggressive cancers. For example, PCas with a high Gleason grade exhibit significantly higher expression of HIF-1α and VEGF and nuclear expression of Snail1 as compared with cancers with a low Gleason grade [166]. Expression of eNOS plus ERβ and of nuclear eNOS plus HIF-2α are indicators of adverse clinical outcome [277].

Interaction with Androgen Receptor and Aryl Hydrocarbon Receptor

In addition to their homo-dimerization or hetero-dimerization with other subtypes or isoforms, ERs interact with other transcription factors. For example, ERβ can interact with the Androgen receptor (AR) [276, 278]. Treatment of the PCa cell line LNCaP with either E2 or androgen triggers the association between ERβ, AR, and Src [276]. A complex consisting of AR and ERβ enables E2-induced activation of AR-regulated genes in the absence of androgens in PCa cells [278]. AR can also interact with ERα [279], and its over-expression inhibits the transcriptional activity of ERα [280, 281]. The antagonistic interplay between estrogenic and androgenic signaling mediated by ERα and AR, respectively, has been demonstrated on a genomic scale in breast cancer [282]. A study has also suggested that the direct interaction between E2 and the androgen receptor accounts for the stimulatory and anti-androgenic actions of E2 on the growth of a breast cancer cell line [283].

The interaction between ERα and aryl hydrocarbon receptor (AhR) exerts physiological effects on different organs [284, 285]. The AhR agonist 2,3,7,8-tetra chlorodibenzo-*p*-dioxin (TCDD) exhibits antiestrogenic activity on the transcriptional activities of ERs [286–288], leading to the inhibition of E2-mediated growth of breast cancer cells by downregulation of E2-induced cell-cycle proteins [287] and also inhibiting E2-induced ERα-mediated upregulation of ERβ mRNA [289]. In contrast, ERα can act as a co-regulator of AhR-mediated transcription activation of CYP1A1 promoter [286, 288, 290]. Recently, AhR has been shown to be a E3 ubiquitin ligase of ERα and AR [291, 292]. The cross talks can be inhibitory to each and cause degradation of both receptors by forming a complex with the proteasome [292, 293].

Although the cross talk between ERs and AR or AhR are well established in other tissues, related studies of the prostate are limited. The highly complicated network of cross talk between ERs, AhR, and AR serves as a good example of an agonist for one transcription factor disrupting other endocrine responses. In interpreting future data, we need to pay attention to the involvement of other signaling pathways in addition to the individual actions of hormones or chemical compounds.

Conclusion

Estrogens and their receptors are implicated in PCa development and tumor progression. Both the traditional estrogen receptors (ERα and ERβ) as well as the alternate receptor GPR30 exert their action via ligand and non-ligand pathways. While the nuclear receptors ERα and ERβ elicit genomic action via the classical ERE, they also cross talk with other enhancers through tethering. Additionally, both receptors have been shown to reside in part in the cell membrane and be activated by various growth factors, leading to the utilization of multiple intracellular signaling for transduction of their action. The non-genomic action of estrogen is further broadened by GPR30, which is a prominent transmembrane receptor. Additional functional diversification of these receptors is afforded by (1) genetic variations that influence activities and stability, (2) alternative splicing generating unique isoforms such as those of ERβ, (3) alternate usage of promoters, (4) cell type specific compartmentation, and (5) distinct differences in ligand affinities for these receptors. These receptors and their isoforms may exert tumor suppressive and tumor-promoting action depending on the context of the cell type, the presence of specific ligands or growth factors, and the tumor grade or stage of progression. This provides a multifaceted signaling circuitry for estrogen during the evolution and progression of the disease. This complexity creates a high degree of challenge for understanding the biology and clinical relevance of these receptors, which should not be viewed in an isolated receptor-by-receptor manner. It also represents exciting opportunities for research in many new fronts and potentials for translating the discoveries to diagnosis/

prognosis and the clinical management of PCa. Recent emergence of new ERα/ERβ agonists/antagonists and GPR30 agonists provide great promise for preventing and treating PCa or in delaying disease progression.

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Chapter 15
Vitamin D and Prostate Cancer

Jung-Sun Kim, Justin M. Roberts, and Nancy L. Weigel

Abstract There is substantial interest in whether vitamin D signaling plays a role in reducing risk for prostate cancer and in its use as a therapeutic target in prostate cancer. Vitamin D is synthesized in the skin through a UVB-mediated reaction and subsequently hydroxylated to form 1,25-dihydroxyvitamin D_3, the ligand for the vitamin D receptor (VDR), a hormone activated transcription factor. Epidemiological studies correlating prostate cancer risk with reduced exposure to sunlight have suggested that vitamin D reduces risk, but the conclusions from studies of vitamin D metabolites have been variable. Similarly, despite promising results in preclinical studies, attempts to target VDR clinically have been less successful. This chapter reviews what is known regarding the actions of VDR in prostate and in prostate cancers and the evidence for activation of VDR as a strategy to reduce risk and/or treat prostate cancer. The chapter summarizes the evidence for a role in reducing prostate cancer risk and discusses the possibility that aberrant vitamin D metabolism contributes to the difficulties in correlating serum vitamin D metabolites with the level of VDR activation in cells. Also discussed are other mechanisms for resistance to the beneficial actions of VDR and strategies to optimize VDR activity.

Introduction

Vitamin D signaling is important for bone and mineral metabolism including the regulation of calcium and phosphate uptake in intestine [1]. Current recommended levels of vitamin D are based on maintaining bone health. There is increasing

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evidence that vitamin D plays a role in many other tissues and that its actions may contribute to the prevention of a variety of diseases including colon and breast cancer [1, 2]. However, the optimal levels of vitamin D to induce these changes likely are higher than those required for maintenance of bone. There is substantial interest in the role of vitamin D signaling in the prevention or treatment of prostate cancer, but the evidence to support a role for vitamin D action in humans is conflicting. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25D) is the ligand for the vitamin D receptor (VDR), a member of the same hormone activated transcription factor family as the androgen receptor [3]. VDR most commonly forms heterodimers with the retinoid X receptors (RXRα, RXRβ, and RXRγ), although there is some evidence for VDR homodimers as well. In response to 1,25D, VDR binds to specific DNA sequences recruiting a series of coactivator complexes to induce transcription. Similar to other nuclear receptors, VDR binding sites are found both upstream and downstream of target genes as well as in introns [4]. VDR also can repress transcription of target genes either through direct binding to DNA, by binding to other proteins or through induction of miRNAs. There are a number of polymorphisms in the VDR gene. One of these, a FokI restriction site in exon 2 (f) results in a 427 amino acid protein; the VDR gene lacking the FokI restriction site (F) produces a 424 amino acid protein, which is thought to be more active than the (f) form [3]. VDR, the RXRs, and the coregulators all are posttranslationally modified; phosphorylation is one of the primary modifications of these proteins. Thus, the activity of VDR is regulated not only by hormone levels, but also by receptor levels and form, the RXR partner, by levels of coregulators, and by cell signaling.

Metabolism of Vitamin D

Metabolism in Normal Tissues

Provitamin D (7-dehydrocholesterol) in the skin is converted to vitamin D_3 (cholecalciferol) through a UVB-mediated reaction as a result of exposure to sunlight (Fig. 15.1). For most people, sunlight is the primary source of vitamin D. Intestinal absorption of vitamin D_3 or vitamin D_2 (plant derived) obtained from dietary sources or supplements provides the remaining vitamin D. Vitamin D enters the blood stream and is taken up by the liver where it is hydroxylated by 27-hydroxylase (CYP27A1) or CYP2R1 to produce 25(OH)D (25D) (calcidiol) [5]. 25D is then hydroxylated by 1α-hydroxylase (CYP27B1) in the kidney to produce the biologically active 1,25D [$1,25(OH)_2D$]. Because 25D is the primary circulating form of vitamin D, it is used as a measure of vitamin D status. Serum 25D is in the nanomolar range (typically up to 80 nM) and has a half-life of about 15 days, whereas 1,25D typically is 1,000-fold lower (picomolar) and has a half-life of 10–20 h [6]. Production of 1,25D in the kidney is tightly regulated by parathyroid hormone (PTH), calcium, phosphorous,

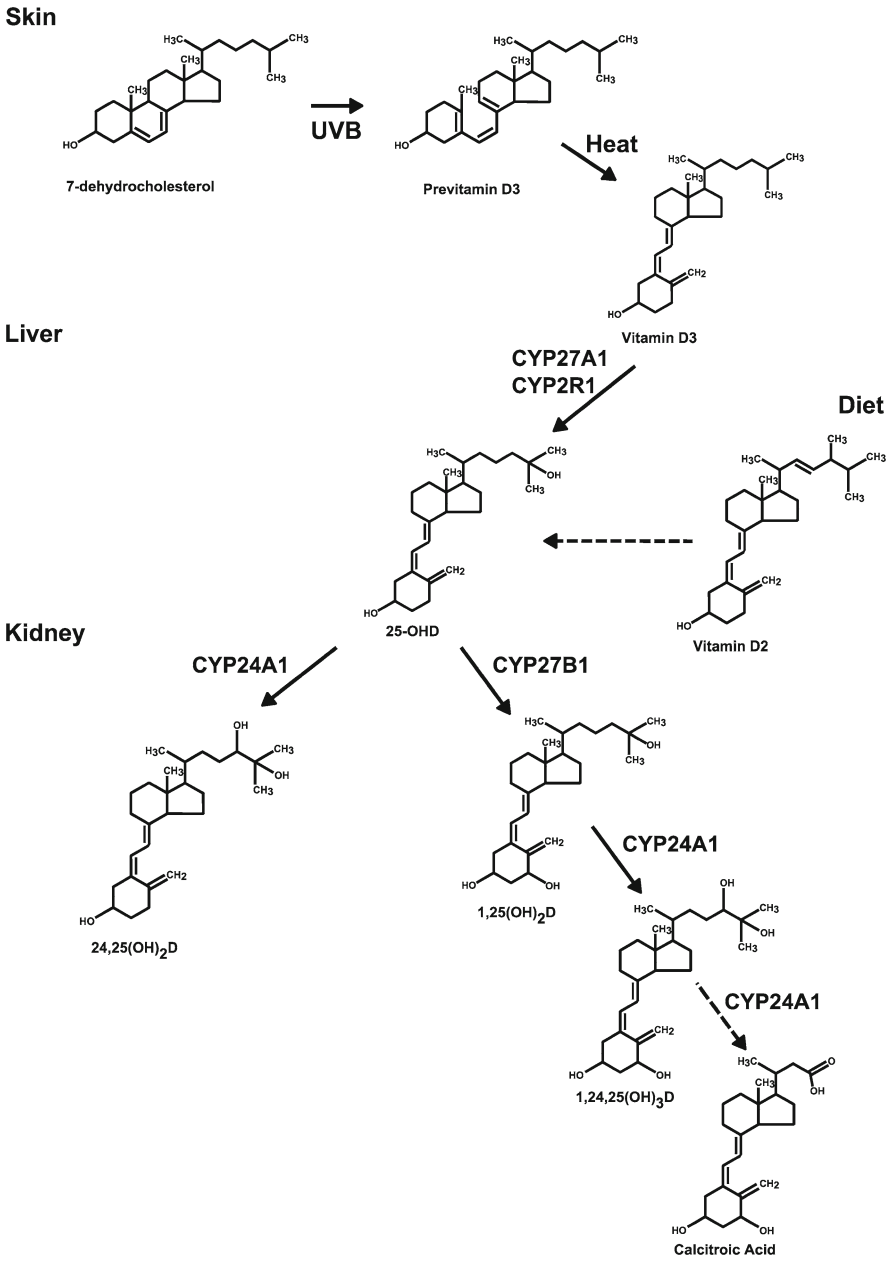

Fig. 15.1 *Vitamin D metabolism*. Exposure of the skin to sunlight results in the photochemical conversion of 7-dehydrocholesterol to previtamin D_3. Previtamin D_3 undergoes isomerization to form vitamin D_3. Hydroxylation of vitamin D_3 and vitamin D_2 by CYP27A1 or CYP2R1 occurs in the liver forming 25-OHD. The next hydroxylation step occurs in the kidney by CYP27B1 forming the biologically active metabolite, $1,25(OH)_2D$. 25-OHD and $1,25(OH)_2D$ are both targets for hydroxylation by CYP24A1 producing $24,25(OH)_2D$ and $1,24,25\ (OH)_3D$, respectively. Calcitroic acid is formed through subsequent metabolism by CYP24A1 and is excreted in the urine

FGF23, and 1,25D [7]. Although the kidney is the primary source of 1,25D, CYP27B1 is expressed in a number of other tissues including prostate. The relative contributions of serum 1,25D and endogenous synthesis of 1,25D to VDR activity are not known. Levels of 1,25D are limited by VDR-dependent induction of 24-hydroxylase (CYP24A1) in the kidney as well as in other tissues. CYP24A1 metabolizes both 25D and 1,25D to less active metabolites ($24,25(OH)_2D$ and $1,24,25(OH)_3D$, respectively). $1,24,25(OH)_3D$ can undergo subsequent oxidations by CYP24A1 to a final product, calcitroic acid, which is excreted in the urine [8]. In the kidney, CYP24A1 is induced by 1,25D and FGF23, and expression is limited by PTH.

Alterations in Metabolism in Cancer

Normal prostate cells express CYP27B1; primary prostate cell cultures and non-transformed prostate cells synthesize sufficient 1,25D to be growth inhibited at levels of 25D similar to the levels of 1,25D required to inhibit growth. In contrast, primary prostate cancer cells and prostate cancer cell lines (DU145, PC3, and LNCaP) have a significant reduction in CYP27B1 activity [9, 10] and respond only to very high levels of 25D consistent with the known weak agonist activity of 25D. CYP27B1 activity is lost through multiple mechanisms in prostate cancer; in prostate cancer cell lines, CYP27B1 RNA is greatly reduced. In the case of DU145 prostate cancer cells, this appears to be due to epigenetic regulation. Treatment of DU145 cells with a combination of histone deacetylase and methyltransferase inhibitors increases both RNA levels and 1,25D metabolites [11]. Factors required for induction of CYP27B1 also may be missing. Studies using luciferase-based CYP27B1 promoter constructs show minimal activity in DU145 and PC-3 cells and no activity in LNCaP cells compared to the activity in normal prostate cells [12]. However, in primary cultures of prostate adenocarcinoma cells, loss of activity apparently is not due to changes in total RNA or protein levels suggesting alternative splicing or posttranslational regulation [13]. Splice variants of CYP27B1 have been reported in several cancers and cell lines although not in prostate cancer [14–18]. Some of the splice variants encode truncated proteins [14] and are less active [18].

Aberrant expression of CYP24A1 limits the ability of cells to respond to 1,25D. CYP24A1 hydroxylates and inactivates both 25D and 1,25D. Thus, this enzyme not only catabolizes 1,25D, but indirectly limits its synthesis. For example, endothelial cells with high endogenous levels of CYP24A1 are resistant to 1,25D-mediated growth inhibition, but depletion of CYP24A1 using siRNA restores sensitivity to 1,25D [19]. Aberrant activation has been suggested as a factor in colon cancer, but this may not occur in prostate cancer. Interestingly, dihydrotestosterone (DHT) reduces VDR-dependent CYP24A1 expression in LNCaP cells [20, 21] suggesting

that aberrant CYP24A1 expression is more likely to be a factor in patients treated with some form of androgen blockade. Androgen receptor (AR) negative DU145 prostate cancer cells express high levels of CYP24A1, and P450 inhibitors that inhibit CYP24A1 activity enhance response to 1,25D [22, 23].

Epidemiology

The evidence supporting a role for vitamin D in reducing risk for prostate cancer is conflicting. On the one hand, the predominant nonfamilial risk factors for prostate cancer are age, race, and geographical location; in each case vitamin D levels are reduced. Production of vitamin D decreases with age [24], the melanin in darker skin limits the efficiency of vitamin D synthesis [25], and populations in northern latitudes tend to have lower levels of vitamin D metabolites [25, 26]. Several observational studies have shown a correlation between higher exposure to sunlight and decreased risk for prostate cancer [27–30] or a decreased risk of death from prostate cancer. In contrast, studies using 25D as a surrogate for levels of VDR activity have shown reduced risk [31], no difference, and even an increased risk [32]. A longitudinal nested case-control study of Nordic men found a U-shaped risk of prostate cancer at both low and high levels of serum 25D, concluding that vitamin D deficiency should be avoided but too much could trigger high catabolism of vitamin D by CYP24A1 [33]. Another study found that high levels of 25D did not alter overall risk, but reduced risk for lethal prostate cancer [34]. There are several limitations in relying on serum 25D measurements. First, these studies typically rely on one or two measurements over the lifetime of the individual and may not be an accurate reflection of overall lifetime levels. Second, as discussed in the metabolism section, serum 25D may not reflect levels of activity in the tumor. One of the limitations of serum-based studies is that these measurements do not reflect levels of 25D or 1,25D in the prostate. This is particularly important when considering 1,25D because it can be synthesized in the prostate and thus serum levels may not reflect tissue levels of 1,25D.

Another factor is the form or expression level of VDR. There have been several polymorphisms described for VDR [3]. Most are not in the coding region, although they may alter expression levels. The FokI polymorphism does change VDR sequence as discussed above. A population-based, case-control study of advanced prostate cancer showed a reduced risk of advanced prostate cancer in cases of high sun exposure and high activity VDR polymorphisms (F/F or F/f) [29]. Other studies have found varying degrees of association and significance of VDR polymorphisms with prostate cancer risk. As is the case for sun exposure and serum based studies, elucidating how these factors interact with each other and contribute collectively and individually to prostate cancer risk is important to our understanding of vitamin D's relationship to prostate cancer etiology.

Responses of Prostate and Prostate Cancer Cells to Vitamin D Signaling

Initial studies showed that 1,25D reduces the growth of most, but not all, prostate cancer cell lines with the response ranging from an almost complete halt in growth, to a modest reduction in cell number [35, 36]. Studies of human primary normal prostate cells as well as primary malignant prostate cells also show that the cells are growth inhibited by 1,25D [37].

In normal tissues, such as the mammary gland, 1,25D often acts as a differentiating agent [38] and thus has multiple targets. Several groups have shown that 1,25D induces a G1 arrest in LNCaP cells [35, 39], and there is some evidence for induction of a small amount of apoptosis [40]. However, other cell lines show an overall decrease in growth without a specific accumulation of cells in G1 [41]. Reductions in invasiveness, secretion of angiogenic factors, etc. also have been reported. Our knowledge of the VDR targets that elicit these changes is summarized below.

Although the evidence that 1,25D can inhibit growth in vitro is compelling, the use of 1,25D or VDR signaling in general as a therapeutic target is limited by hypercalcemia as a result of too much VDR action in intestine and bone. Dietary vitamin D regulates growth of normal mouse prostate epithelial cells [42]. Subnormal levels of vitamin D that result in somewhat deficient serum levels of 25D (25 nM) in mice results in a two fold increase in Ki67 staining and reduces apoptosis relative to cells in mice provided with adequate levels of vitamin D. High levels of dietary vitamin D also reduce prostatic intraepithelial neoplasia formation (PIN) in the TgAPT(121) [42] mouse model, and 1,25D can reduce PIN formation in NKX3.1/ PTEN mutant mice [43]. Increased dietary vitamin D also reduces the growth of PC-3 prostate cancer cell xenografts under conditions that elevate serum 1,25D [44]. Another approach to activating VDR without inducing hypercalcemia is to use less calcemic analogs of 1,25D. These compounds are less potent in inducing targets in intestine and bone that cause hypercalcemia, yet retain the ability to inhibit prostate cancer cell growth. EB1089 (seocalcitol) inhibits LNCaP xenograft growth without causing hypercalcemia [45] as does a non-secosteroidal VDR agonist [46] and a newer analog, inecalcitol [47]. EB1089 also reduces PTHrP-induced metastasis [48]; activation of VDR also has been reported to inhibit growth of osteoblastic bone lesions [49].

VDR Actions and Targets in Prostate Cancer

Introduction

Prostate tumors contain a heterogeneous mixture of mutations that can alter their response to treatment. One of the highest frequency genomic alterations in prostate cancer is the genomic translocation that results in the fusion of the

androgen-regulated TMPRSS2 promoter to the coding region of an oncogenic ETS factor such as ERG [50]. These translocations are found in >50 % of prostate cancers. Another frequent change is loss of PTEN [51], which results in elevated PI-3K and Akt signaling. The combination of these two changes is sufficient to induce prostate cancer in mouse models [52]. Over-expression of c-Myc is also common in prostate cancer where overexpression of c-Myc is sufficient to induce PIN and/or prostate cancer in mouse models [53–55]. Similar to other cancers, some prostate tumors lose the tumor suppressors, p53 and Retinoblastoma protein (Rb), at some point in progression. Much of our knowledge of VDR action at the molecular level is derived from the characterization of the LNCaP prostate cancer cell line and its castration-resistant derivative, C4-2 cells [56]. These cells are AR positive and AR dependent for growth although the C4-2 cells will grow in androgen-depleted medium [56]. These cells are PTEN negative [57], but retain functional p53 [58] and Rb. They do not contain a genomic TMPRSS2:ETS factor translocation [50]. The other cell lines most commonly used to study VDR action are the PC-3 and DU145 cells. Both lines are AR negative [58], which is not typical of most prostate cancers. PC-3 cells are PTEN negative [59] and lack functional p53 [58]. DU145 cells are PTEN positive [59] but lack functional p53 [58] and Rb [60]. The androgen-dependent VCAP cell line is the only widely used prostate cancer cell line that contains a TMPRSS2:ETS factor genomic translocation [50]. It is PTEN positive and has mutant p53 [58].

Cell Cycle

One of the primary mechanisms for 1,25D-mediated cell growth inhibition is regulation of cell cycle associated gene expression both transcriptionally and posttranscriptionally (translation and/or protein stability). 1,25D causes accumulation of primary human prostate epithelial cells and LNCaP cells in the G0/G1 phase of the cell cycle [35, 61]. PC-3 cells, which are p53 negative, are growth inhibited by 1,25D, but do not accumulate in G1 [41]. However, the differential responsiveness is unlikely due to the lack of p53. Expression of a dominant negative p53 in LNCaP cells prevents the G0 accumulation as judged by loss of Ki67 staining, but does not block the G1 arrest or overall level of growth inhibition [62].

The requirement for a functional Rb for 1,25D-mediated growth inhibition appears to be cell line specific. Rb regulates progression of cells from G1 to S phase (Fig. 15.2). When Rb is active, it binds to and inactivates E2F family members, transcription factors required for induction of genes that are critical for the progression to S phase [63, 64]. Cyclin D/cyclin-dependent kinase (cdk) 4/6 and cyclin E/cdk2 complexes phosphorylate and inactivate Rb causing release and activation of E2F [65]. 1,25D treatment alters expression of a number of cell cycle proteins to prevent Rb inactivation (Fig. 15.2). Treatment with 1,25D increases the expression of cdk inhibitors, p21 and p27 [39, 61]. Although p21 has been shown to be regulated primarily at the protein level in LNCaP cells, three functional VDREs within

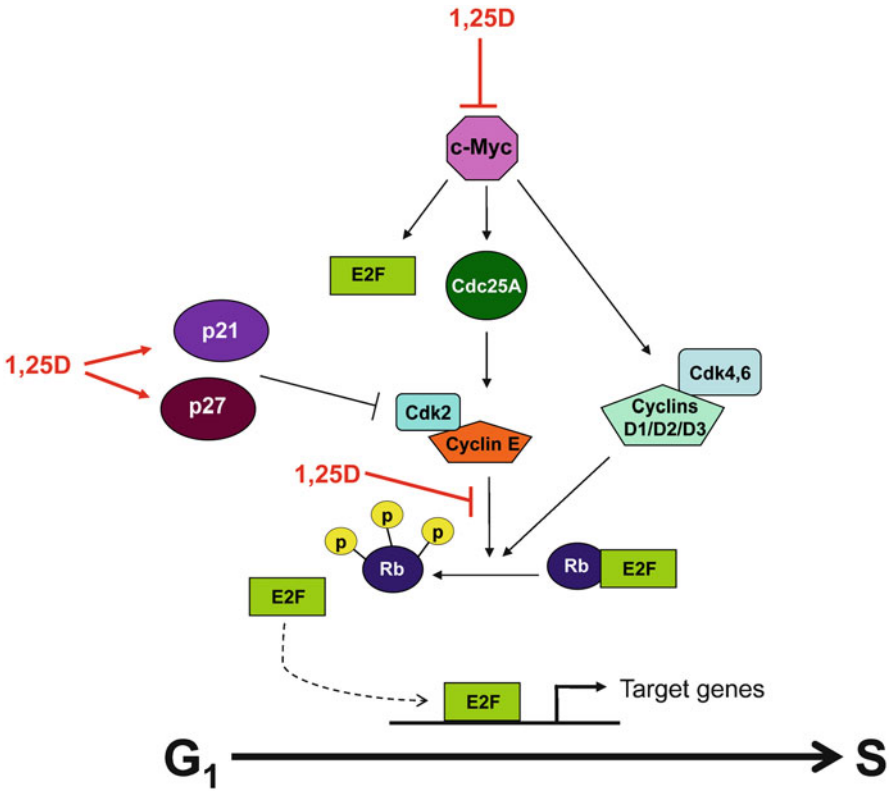

Fig. 15.2 *Effect of 1,25D on the cell cycle.* 1,25D regulates the expression of cell cycle associated genes and their activities through multiple mechanisms. C-Myc regulates expression of numerous cell cycle associated genes required for G1 to S phase transition. In C4-2 cells, 1,25D downregulates c-Myc transcription and reduces the half-life of c-Myc protein. This decrease in c-Myc level leads to reduction in E2Fs, cdc25A, and cyclins. Independently, 1,25D induces p21 and p27 expression which leads to cdk2 inactivation, reduced phosphorylation of Rb, and increased Rb activity. This results in sequestering of E2F by Rb and causes cell cycle arrest

the p21 promoter were identified in immortalized non-transformed prostate epithelial RWPE1 cells [61, 66]. A microarray study in RWPE1 cells has revealed numerous cell cycle associated genes regulated by 1,25D. However, expression of many genes is altered after a relatively long term of treatment (24–48 h) suggesting that regulation of some of these may be secondary to cell cycle arrest [67]. In LNCaP cells, 1,25D promotes mislocalization of cdk2 to the cytoplasm, reduces the levels of an activating phosphorylation, and decreases cdk2 activity, thus reducing Rb inactivation and cell cycle progression [68]. Depletion of Rb reduces the growth inhibitory effect of 1,25D in LNCaP cells [68]. On the other hand, depletion of Rb in the isogenic C4-2 cell line does not prevent 1,25D-mediated growth inhibition suggesting an Rb-independent mechanism for inducing cell cycle arrest [69].

C-Myc is an oncogenic transcription factor, which plays multiple roles in the promotion of cell cycle progression [70, 71]. C-Myc directly induces expression of Cdc25A, a phosphatase whose activity is necessary for activation of cdk2 C-Myc also induces expression of E2Fs. 1,25D reduces c-Myc expression in multiple cell lines. In C4-2 cells, 1,25D reduces c-Myc RNA expression and further reduces protein expression by decreasing c-Myc protein half-life [72]. Furthermore, when c-Myc expression is reduced by siRNA to the level caused by 1,25D treatment, the inhibition of proliferation and accumulation in the G1 phase is similar to the level observed as a result of 1,25D treatment [72]. The extensive downregulation of c-Myc resulting in reduced E2F levels likely explains the Rb-independent growth inhibition in these cells [69]. Expression of c-Myc is regulated at many levels including transcription initiation, elongation, RNA stability, protein stability, and posttranslational modification. In these cells, 1,25D treatment begins to reduce c-Myc expression prior to a detectable inhibition of proliferation suggesting that the downregulation is a direct, early effect of 1,25D treatment [69]. VDR binds directly to sequences upstream of the c-Myc gene as has been shown through chromatin immunoprecipitation (ChIP)-Seq studies in the colorectal cancer cell line LS180 [73]. 1,25D promotes degradation of c-Myc protein at least in part by phosphorylation of the c-Myc protein at T58, which is a signal for protein degradation [72]. There also may be other mechanisms by which 1,25D inhibits c-Myc expression or activity.

Other proteins have also been shown to play a role in 1,25D-mediated growth inhibition. CCAAT/enhancer-binding protein delta plays a role in 1,25D-mediated growth inhibition of LNCaP cells [74]. Depleting CCAAT/enhancer-binding protein delta reduces the ability of 1,25D to inhibit growth. Interestingly, CCAAT/enhancer-binding protein delta expression is inhibited by c-Myc [75]. Thus its induction by 1,25D may be a result of 1,25D-mediated downregulation of c-Myc. GADD45gamma is also induced by 1,25D in LNCaP cells, but not in a variant of LNCaP that was selected for resistance to 1,25D-mediated growth inhibition. Expression of GADD45gamma in the 1,25D-resistant cell line inhibited growth [76].

Apoptosis

1,25D or its less calcemic analog (EB1089) induces a small amount of apoptosis [40, 77]. 1,25D-mediated apoptosis is accompanied by decreased expression of anti-apototic Bcl-2 and Bcl-X(L) proteins in LNCaP and ALVA31 cells. Furthermore, overexpression of Bcl-2 is sufficient to block 1,25D-mediated apoptosis [40, 78]. 1,25D can also cause apoptosis in LNCaP cells, which contain dominant negative p53 suggesting that 1,25D-mediated induction of apoptosis is independent of p53 [62]. Recently developed calcipotrine-derived vitamin D_3 analogs, BGP-13 and BGP-15, also inhibit cell growth and induce apoptosis in LNCaP cells [79]. Collectively, these studies suggest that the induction of the apoptotic pathway

contributes to the 1,25D-mediated growth inhibition in prostate cancer cells. However, the extent of this contribution and the mechanisms by which 1,25D mediates apoptosis are still not fully understood.

Regulation of Signaling Pathways

There is good evidence that 1,25D acts in part by regulating components of signaling pathways. Transforming growth factor beta (TGFβ) is an antiproliferative and pro-apoptotic factor in normal and early stage malignant epithelial cells [80, 81]. On the other hand, TGFβ can promote growth, angiogenesis, and migration in malignant prostate tissues and prostate stroma cells indicating the cell-specific actions of TGFβ [82]. TGFβ binds to two cell surface receptors Tβ-RI and Tβ-RII leading to phosphorylation of Smad2/3 to regulate target gene expression [83]. 1,25D activates the TGFβ signaling pathway at least in part by induction of TGFβ expression. This pathway is important for 1,25D-mediated inhibition of growth in PC-3 cells [84]. Interestingly, a neutralizing antibody against TGFβ1 and TGFβ2 abolishes the growth inhibitory effect of 1,25D in PC-3 cells indicating that the TGFβ pathway is required for 1,25D-mediated growth inhibition [84]. In contrast, LNCaP cells lack functional TGFβ receptor and are resistant to TGFβ.

1,25D also can have effects on insulin-like growth factor (IGF) signaling, a pathway that promotes proliferation and limits apoptosis. Increased IGF signaling is associated with an increased risk for prostate cancer [85, 86]. IGF-binding protein 3 (IGFBP3) limits IGF1 and IGF2 actions by binding to and interfering with the ability of these proteins to interact with cell surface receptors. The importance of IGFBP3 is supported by mouse studies. IGFBP3 knockout mice crossed with a c-Myc driven prostate cancer model increased metastasis, and cell lines derived from these tumors exhibited a more aggressive phenotype with increases in proliferation, invasion, and anchorage-independent growth [87]. Interestingly, microarray studies showed increased expression of IGFBP3 mRNA in LNCaP and RWPE1 cells treated with 1,25D [67, 88]. Thus, 1,25D-mediated regulation appears to be direct since a putative VDRE has been identified in the IGFBP3 promoter by electrophoretic mobility-shift assay (EMSA) [89], and binding sites have been identified by ChIP [89]. The necessity for IGFBP3 induction for 1,25D-mediated cell growth inhibition depends on serum conditions [90, 91]. In serum free media containing serum replacement supplement, depletion of IGFBP3 using antisense oligonucleotides or addition of neutralizing antibody against IGFBP3 abolishes the growth inhibitory role of 1,25D in LNCaP cells [90]. In fetal bovine serum (FBS) containing media, depletion of IGFBP3 using siRNA does not affect 1,25D-mediated growth inhibition in LNCaP cells [91]. FBS contains other growth factors that contribute to cell growth. Therefore, cell growth may heavily depend on IGF signaling in the absence of FBS, whereas other signaling pathways may compensate for cell growth in the presence of FBS.

Parathyroid hormone-related protein (PTHrP) enhances cell proliferation, migration, and anchorage-independent growth of prostate cancer cells [48, 92]. 1,25D

reduces PTHrP expression via multiple mechanisms. 1,25D reduces PTHrP RNA expression through a negative VDRE in LNCaP cells and reduces PTHrP RNA as well as protein half-life in PC-3 cells [93, 94]. PTHrP increases expression of the pro-invasive and pro-proliferative integrin α6/β4. Depletion of PTHrP using siRNA significantly reduces the ability of 1,25D to inhibit integrin α6/β4 expression, and overexpressing PTHrP significantly attenuates the ability of 1,25D to regulate integrin α6/β4 expression. These depletion and overexpression studies indicate that 1,25D-mediated repression of PTHrP expression plays a role in the 1,25D-mediated reduction in integrin α6/β4 expression [95]. EB1089 inhibits xenograft growth and bone metastasis of PTHrP overexpressing C4-2 cells in nude mice suggesting that VDR action would be beneficial in patients with PTHrP over-expressing prostate tumors [48].

DNA Damage/Reactive Oxygen Species

DNA damage and/or a malfunctioning of DNA repair mechanism can lead to carcinogenesis. Moreover, oxidative stress arising from reactive oxygen species (ROS) can cause DNA damage. There is some evidence for a protective role of VDR against DNA damage in colon [96, 97]. The amount of 8-hydroxy-2′-deoxyguanosine, a marker for oxidative stress, is significantly elevated in colon epithelium of VDR knockout mice compared to WT mice, and is reduced in colon epithelium of patients receiving 800 I.U. of vitamin D_3 suggesting that VDR has anti-DNA damage activity [96, 97]. In nonmalignant prostate cells, 1,25D directly induces glucose-6-phosphate dehydrogenase (G6PD), an enzyme that protects cells from ROS, but does not induce G6PD in cancer cell lines. Moreover, the 1,25D-mediated regulation of G6PD is required for VDR's protective action against H_2O_2 induced DNA damage in BPH1 and RWPE1 cells [98]. 1,25D also regulates expression of other genes involved in regulating ROS levels including thioredoxin reductase I (TXNRDI) and superoxide dismutase (SOD) in human prostatic epithelial cells [99]. 1,25D induces ATM and RAD50 in NMU treated BPH-1 cells, and overall DNA repair activity is increased [100]. As discussed above, CCAAT/enhancer binding protein delta is induced by 1,25D. Recent studies suggest that it also plays a role in DNA repair [101]. Thus, although there are limited studies, current evidence suggests a role for VDR in protecting cells against ROS and DNA damage.

Angiogenesis

Angiogenesis is a critical limiting step for tumor growth and metastasis. Several studies have suggested a promising role for VDR in inhibiting angiogenesis. 1,25D inhibits angiogenesis both by directly inhibiting endothelial cell growth and by inhibiting expression of pro-angiogenic factors [102, 103]. When prostate tumors from TRAMP2 mice are subcutaneously injected into VDR knockout mice, the

tumor growth and the extent of vasculature formation are significantly higher compared to WT mice [104].

1,25D inhibits expression of multiple pro-angiogenic factors. Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor involved in the initiation of blood vessel formation. 1,25D reduces VEGF expression in normal prostate epithelial cells. Two functional VDREs have been identified in the VEGF promoter supporting a direct regulation of VEGF expression by VDR [105, 106]. 1,25D also can indirectly regulate VEGF expression by inhibiting hypoxia-inducible factor-1 (HIF-1) expression because VEGF is an HIF-1 target gene [107].

Interleukin-8 (IL-8) is a chemotactic factor also involved in promoting angiogenesis and cell growth. Incubation of endothelial cells with recombinant IL-8 enhances cell proliferation and induces capillary tube organization indicative of angiogenesis [108]. Prostate tumors exhibit elevated levels of IL-8 highlighting the importance of targeting IL-8. Interestingly, 1,25D inhibits IL-8 mRNA and protein expression in a number of prostate epithelial cells lines including RWPE1, LNCaP, PC-3, and DU145. Furthermore, 1,25D or an IL-8 neutralizing antibody reduces the HUVEC tube formation induced by LNCaP, PC-3, and DU145 cells [109]. Thus, repression of IL-8 is likely a factor in VDR-mediated inhibition of angiogenesis.

Inflammation/Prostaglandin

Chronic inflammation increases risk for prostate cancer and both infection and diet are thought to contribute to the inflammation of the prostate [110]. These factors can lead to an increase in oxidative damage as well as an increase in prostaglandins. NSAIDs can modestly reduce risk for prostate cancer. Based on gene expression studies, 1,25D may be able to act as an anti-inflammatory agent in prostate cancer. One of the key inflammatory pathways is the prostaglandin (PG) signaling pathway, which promotes cancer development and progression. Cyclooxygenase-2 (COX-2) converts arachidonic acid to PGs. Several reports have described overexpression of COX-2 in prostate cancer [111–114]. Another key enzyme in the PG pathway is 15-prostaglandin dehydrogenase (15-PGDH). 15-PGDH inhibits the PG pathway by converting PG into its inactive 15-keto form. In summary, 1,25D inhibits the activity of the PG pathway via three different mechanisms: inhibiting COX-2 expression, inducing 15-PGDH, and inhibiting expression of PG receptors [88, 115, 116].

Clinical Trials Targeting VDR Action

Promising results from preclinical models have led to clinical trials for prostate cancer with high doses of vitamin D, 1,25D, or 1,25D analogs. Because it is not feasible to measure tumor burden especially in patients with metastases, the change

in serum prostate specific antigen (PSA) with time is used as a surrogate for changes in tumor burden in vitamin D clinical trials. Daily supplementation of vitamin D_3 soft gels containing 4,000 international units (i.u.) for 1 year in low risk prostate cancer led to a significant decrease in positive biopsy cores without a significant change in serum PSA levels [117]. On the other hand, a small pilot clinical trial with open label, nonrandomized treatment with slowly increasing doses of 1,25D for 6–15 months showed a significant decrease in the rate of PSA rise in six out of seven patients having early recurrent prostate cancer [118]. Another trial with a high weekly dose of 1,25D in 22 patients showed no PSA response in 50 % of the patients, reduced PSA in three patients, and increased PSA doubling time in three patients [119]. It is important to note that none of these 1,25D trials reached the primary efficacy endpoint which is 50 % reduction in serum PSA.

One of the conventional treatments for hormone refractory prostate cancer is the chemotherapeutic agent, docetaxel. Multiple combination clinical trials of 1,25D with docetaxel have been performed. When 1,25D was given in combination with docetaxel in metastatic androgen-independent prostate cancer, 22 out of 37 patients exhibited greater than 75 % reduction in serum PSA level [120]. However, in a much larger clinical trial of 250 patients, a combination of DN-101 (a recently developed high dose formulation of 1,25D) with docetaxel did not result in significant differences in serum PSA. Nonetheless, this study suggested increased survival in the DN-101/docetaxel arm compared to the placebo/docetaxel arm [121]. A subsequent phase III study (ASCENT-2) showed increased death in the DN-1010 + docetaxel arm compared with the docetaxel only arm. One criticism of this trial was that the docetaxel treatment regimes were not the same in the two arms [122]. Unfortunately, there is no assay for activation of VDR in the tumors, so the question of whether there was insufficient VDR activation to produce the desired results or whether VDR activation is insufficient to inhibit tumor growth remains unresolved. Because of side effects, higher levels of 1,25D cannot be used, but less calcemic analogs may be a useful alternative.

Mechanisms for Resistance to 1,25D

The response of prostate cancer cells to 1,25D-mediated growth inhibition ranges from strongly growth inhibited (LNCaP) to little or no growth inhibition (DU145) [36]. Resistance to 1,25D is multifactorial. In some cases there are changes that inhibit overall VDR activity. In others, VDR is functional but there are downstream alterations that prevent VDR-mediated growth inhibition. Changes that reduce overall VDR activity include altered metabolism as discussed earlier. A second mechanism for reducing VDR activity is p42/p44 MAPK-dependent phosphorylation of RXRα either at Ser260 or at sites in the amino-terminal transactivation domain resulting in reduced coactivator recruitment [123, 124]. Higher levels of the corepressor, SMRT, and histone deacetylation have also been implicated in reducing VDR activity in prostate cancer [125]. SNAIL and SLUG reduce VDR

expression and activity in colon cancer and in osteosarcoma [126, 127]. Whether they play a similar role in prostate cancer has not yet been determined.

In addition to direct inhibition of VDR activity, other factors have been implicated in limiting the growth inhibitory response. Expression of SV40 T antigen does not block VDR-mediated induction of CYP24A1, but does block VDR-mediated growth inhibition [128]. The SV40 large T antigen inactivates p53 and Rb. However, p53 negative cells remain responsive to 1,25D. The role of Rb in 1,25D-mediated growth inhibition may be cell line specific. Rb is required for VDR-mediated growth inhibition in LNCaP cells [68], but not in the closely related C4-2 cells [69]. One of the primary functions of Rb is to sequester E2F transcription factors. In C4-2 cells, 1,25D downregulates c-Myc resulting in downregulation of E2F [69]. Thus, E2F activity is reduced by alternate means. The large T antigen likely has other targets and small t antigen sequesters PP2A. Thus, there are additional mechanisms by which SV40 can block 1,25D-mediated growth inhibition.

The specific response of prostate cancer cells to 1,25D also can depend on other genetic changes. The majority of prostate cancers contain genomic translocations that link the promoter of the androgen-regulated TMPRSS2 gene to the coding region of an oncogenic ETS factor, most frequently ERG [50]. Of the available prostate cancer cell lines, only VCaP and the closely related DuCaP line express this fusion [50]. Interestingly, 1,25D induces the TMPRSS2 gene and thus induces the fusion. However, these cells are growth inhibited by 1,25D suggesting that 1,25D has compensatory growth inhibitory actions [129].

Summary

Epidemiological studies showing an inverse correlation between prostate cancer and geography/exposure to sunlight suggest that VDR signaling reduces risk for prostate cancer. However, the correlations of serum 25D levels as a surrogate for VDR activity with prostate cancer risk are not consistent. One of the unknown factors is whether circulating 1,25D is responsible for VDR activity in prostate and prostate tumors. Normal prostate cells can synthesize 1,25D, so higher levels of 25D may lead to higher levels of tissue 1,25D. In contrast, prostate cancer cells lose the ability to produce 1,25D. Thus, if circulating 1,25D determines the level of VDR activation, the risk should be increased at low serum 25D, reduced at moderate levels, but could increase at high levels in part due to inhibition of 1,25D synthesis [33]. The actions of VDR suggest that 1,25D should reduce risk for prostate cancer. Insufficient dietary vitamin D leads to an increase in proliferation and a decrease in apoptosis in normal mouse prostate [42]. In vitro, human prostate cell growth also is inhibited by 1,25D. Moreover, 1,25D is anti-inflammatory, can reduce ROS levels, and facilitate DNA repair. All of these actions likely would reduce the likelihood of the genomic changes required to transform cells.

Similarly, 1,25D inhibits growth of prostate cancer cells in vitro, and less calcemic VDR agonists inhibit growth of LNCaP xenografts in vivo. Many, but not

all, of the VDR dependent changes in gene expression would be expected to inhibit human tumor growth and/or sensitize tumors to other treatments. For example, 1,25D downregulates c-Myc, a driving factor in prostate cancer. Overexpression of Bcl-2 has been associated with CRPC and resistance to apoptosis, and 1,25D reduces Bcl-2 induced expression. 1,25D also reduces angiogenic factors and IGFBP-3, which limits IGF signaling. In contrast to the in vitro and preclinical studies, the results of clinical trials have been disappointing. There are a number of possibilities for these discrepancies. First, most of the in vitro studies are performed with relatively high levels of 1,25D; most of the successful xenograft studies have used less calcemic 1,25D analogs. The clinical trials, for the most part, have used various formulations of 1,25D. Thus, there may be insufficient VDR activation in the tumors due to the dose limiting side effects of high levels of 1,25D, and less calcemic analogs may provide a better response. A second possibility is that response is heterogeneous and that clinical trials have not considered specific genetic changes in the tumors. 1,25D can increase expression of the TMPRSS2:ETS factor translocation. The translocation is highly expressed in the absence of 1,25D, and it is not known whether treatment with 1,25D yields additional ETS factor activity. However, it is possible that translocation negative patients would preferentially benefit from VDR activation. A third consideration is the type of patient in the clinical trials. Most of the clinical trials have utilized a castration-resistant prostate cancer population, which is resistant to virtually all treatments. Activation of VDR may be more beneficial at an earlier stage, for example, when androgen blockade is first administered. In summary, the preclinical studies suggest that VDR activity should reduce risk for prostate cancer and VDR should be considered as a therapeutic target in prostate cancer. Metabolic changes in tumors may limit the beneficial actions of dietary vitamin D. A better understanding of the actions of VDR in various sub-types of prostate cancer and more potent VDR agonists with a greater window between the levels required to activate tumor VDR and those that cause hypercalcemia should enhance the utility of VDR as a therapeutic target in prostate cancer.

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Chapter 16
HDAC6 Regulation of Androgen Signaling in Prostate Cancer

Junkui Ai and Zhou Wang

Abstract Prostate cancer is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer death in the male population in the USA and other developed nations. Initially, prostate cancer is dependent on androgens for growth, which provides a basis for using androgen deprivation therapy to treat locally advanced or metastatic disease. However, prostate cancer almost always progresses to a castration-resistant phenotype for which few treatment options with improved overall survival are available at present. Multiple studies have shown that active androgen receptor (AR) signaling is still required for development, growth, and progression of castration-resistant prostate cancer (CRPC). Thus, targeting AR signaling, particularly androgen-independent activation of AR signaling, in CRPC may result in novel therapeutic strategies. In this chapter, we summarize recent findings on the regulation of AR signaling by histone deacetylase 6 (HDAC6) and discuss the potential mechanisms by which HDAC6 influences androgen signaling in prostate cancer. We also discuss the potential of targeting HDAC6 in prostate cancer treatment.

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Introduction

Prostate Cancer

Prostate cancer is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer death in the male population in the USA and other developed nations. The American Cancer Society estimates that 241,740 men will be diagnosed with prostate cancer and 28,170 will die from this disease in 2012 [1–4]. The vast majority of prostate cancers are diagnosed in the early stages, which are androgen dependent. Thus, most patients respond favorably to androgen ablation, the standard form of treatment for locally advanced or metastatic disease [4]. Though initially the disease displays a relatively indolent course in most patients [2], prostate cancer almost always progresses and becomes resistant to androgen ablation therapy and resumes growth [5–8]. Following relapse after castration, patients with metastatic prostate cancer have few treatment options that are associated with improved overall survival [9–11]. These patients have a median progression-free survival ranging from 12 to 30 months after treatment is initiated [12, 13]. When the state of castration resistance eventually emerges, the median overall survival is only 8–16 months from the time of its appearance [12, 13]. Therefore, it is important to develop new options for treatment of patients with castration-resistant prostate cancer (CRPC).

Androgens and AR Signaling in Prostate Cancer

Androgens and AR signaling pathways are commonly considered as the main oncogenic drivers of prostate carcinogenesis [14]. The major circulating androgen is testosterone, of which 90–95 % is synthesized in Leydig cells of the testis, with only 5–10 % coming from the adrenals [15]. Upon release into circulation, testosterone associates with sex hormone-binding globulin (SHBG), which shepards androgens through the circulatory system to its target tissues like the prostate. Once testosterone enters prostate cells it is converted to dihydrotestosterone (DHT) by 5 alpha reductase enzymes. DHT is the primary androgen in prostate tissues as it has a slower dissociation rate from the AR and exhibits more potent biological effects.

Prostate cancer cells require androgens for both growth and survival. Removal of androgens triggers apoptosis in both normal prostate cells and malignant prostate cells [16]. The growth of prostate cancer in patients also depends on androgens, which stimulate proliferation and inhibit apoptosis. Androgen ablation causes death of prostate cancer cells at a rate greater than that of proliferation. But it is rarely curative. One of the possible mechanisms is the selection pressure leading to the survival of some tumor clones and establishment of the castration-resistant state. Another possible mechanism is an AR signaling de novo [17].

Castration resistance requires active androgen signaling that is mediated by AR via the over-expression, mutation, and/or posttranslational modification of AR and/or its coactivators [5, 8, 18–25]. Most patients relapse with recurrent tumors, which reexpress active AR signaling as indicated by continuous target gene expression, despite having castrate levels of androgens [5]. The activation of AR signaling in the castrate patient (testosterone $\leq$ 50 ng/dL) can be attributed to mechanisms that are mediated by the AR—such in situ androgen production by the tumor and amplification of AR protein—or those that bypass it such as over-expression of coactivators and transactivators [26]. Hypersensitization to ligand may also activate AR in CRPC cells. Grogery et al. found that AR is highly expressed, more stable, and localized to the nucleus in the absence of androgen in recurrent prostate cancer cell lines and in recurrent CWR22 human tumor xenografts after prolonged periods of castration. This increased stability of AR is associated with its hypersensitivity to very low androgen levels [27]. The involvement of active AR signaling in the regulation of CRPC cell proliferation has important implications for the development of new treatment strategies for CRPC.

AR belongs to the nuclear steroid receptor superfamily [19]. It is located on the X chromosome. The AR protein structure is very similar to other steroid receptors. It contains four functional regions: an amino terminal transcriptional activation domain, a well-conserved DNA-binding domain, a hinge region containing a nuclear localization signal, and a carboxy-terminal ligand-binding domain [28]. Without ligand binding, AR is inactive and sequestered predominantly in the cytoplasm complexed with heat shock proteins, molecular chaperones, and immunophilins, which facilitate conformation of high affinity for androgens. Upon androgen binding, ARs dissociate from the complex, dimerize, become phosphorylated, translocate into the nucleus, recruit coactivators, and bind to androgen response elements in the regulatory regions of various androgen responsive genes [14, 29–32].

Targeting AR Signaling in Prostate Cancer Therapy

All current antagonists AR act by competing with androgens for binding the hormone-binding pocket. This mechanism of action exploits the dependence of AR on hormone activation in androgen dependent-prostate cancer. In CRPC, cell growth is much less dependent on androgen, but it is still dependent on active AR, e.g., AR signaling is still active. There is a growing focus on the role of the AR in the context of "androgen-independent" signaling during disease progression [33–36]. Thus, clarifying the mechanisms of androgen-independent activation and regulation of AR signaling in CRPC will provide novel strategies for therapy.

Indeed, drug development has focused on the AR signaling pathway at many points in CRPC patients. Therapies include novel and potent antagonists of androgen binding to AR, inhibitors of androgen synthesis, small molecule tyrosine kinase inhibitors, or mammalian target of rapamycin (MTOR) inhibitors. Targeting of AR cofactors, including Hsp90 and histone deacetylases (HDACs), is also being explored [37].

Hsp90 Regulation of the AR Signaling Pathway

Activation of AR signaling is a dynamic process. Molecular chaperones are required for AR to achieve an appropriate conformation, which is essential for stabilization that is optimal for androgen binding [38, 39]. Heat shock protein 90 (Hsp90) plays a central role in the formation of a multichapterone complex [40–42]. Initially, it was found that hsp90 associates with various nuclear receptors in vivo [43]. Later on, studies reported that Hsp90 also has negligible interaction with ligand-free nuclear receptors in vivo, and addition of ligand further abolishes that interaction [41].

Hsp90 is an ATP-utilizing chaperone that interacts transiently with the ligand-binding domain of AR to stabilize a conformation appropriate for androgen binding [42]. Additionally, accessory proteins termed cochaperones facilitate or stabilize changes in Hsp90 conformation and ATPase activity [39]. All of these accessory proteins are bound to the receptor indirectly via Hsp90 [44, 45]. Hsp90 regulates the client protein half-life by forming conformation-dependent higher order chaperone complexes [46]. Hsp90 is required for the acquisition of an active conformation in agonist-bound AR to regulate nuclear transfer, nuclear matrix binding, and transcriptional activity. Pure anti-androgens block the transconformational change of AR in an intermediary complex that is unable to acquire an active conformation or dissociate from hsp90. Hsp90 inhibition prevents the ligand-dependent nuclear translocation of AR, suggesting a role for Hsp90 in the nuclear import of AR [47].

Using castration-resistant C4-2 cells, which were generated through multiple stages of co-culture of androgen-sensitive LNCaP prostate cancer cells with human bone fibroblast MS cells in castrated male athymic mice, as a model for CRPC [48], recent studies [36] have demonstrated an increased nuclear localization of a GFP-tagged AR in the absence of hormone in C4-2 cells compared to parental LNCaP cells. Analysis of AR mutants that are impaired in ligand-binding indicates that the nuclear localization of AR in C4-2 cells can be truly androgen independent. The hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), inhibits basal PSA expression and disrupts the ligand-independent nuclear localization of AR at doses much lower than that required to inhibit androgen-induced nuclear import. Thus, Hsp90 appears to be a key regulator of ligand-independent nuclear localization and activation of AR in CRPC cells [36].

Hsp90 Regulation by Histone Deacetylase 6

Histone deacetylase 6 (HDAC6) is a member of the HDAC family. HDACs comprise a group of enzymes that are responsible for the removal of acetyl groups from an ε-*N*-acetyl lysine amino acid. The organization and packaging of eukaryotic DNA in chromatin structure is achieved through the addition of the core histones H2A, H2B, H3, and H4. Modifications in these core histones lead to conformational

changes in chromatin [49, 50]. The level of histone acetylation influences transcription activity: acetylation induces an open chromatin confirmation that allows access of the transcription machinery to promoters. Chromatin acetylation correlates with transcriptional activity (euchromatin), whereas deacetylation correlates with gene silencing. HDACs are also involved in deacetylation of nonnuclear proteins like Hsp90 [51, 52].

HDACs are grouped into three classes based on their primary homology to three *Saccharomyces cerevisiae* HDACs. The class I HDACs, which include HDAC1, HDAC2, HDAC3, and HDAC8, are most related to the yeast transcriptional regulator yRPD3. Class II HDACs, which include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10, share domains similar to yHDA1. HDAC11 is most closely related to class I HDACs; however, no classification has been given due to its low sequence similarity [53–55].

The human HDAC6 gene is located on Xp11.23 and was first cloned by two different groups [56, 57]. It encodes a very large protein with 1,216 amino acids [56, 58]. Most HDACs are located in the nucleus; however, class II HDACs are able to translocate to the cytoplasm. HDAC6 is located predominately in the cytoplasm [54, 59] and is also found in perinuclear and leading-edge subcellular regions [51]. HDAC6 is a unique member of class II because it possesses two separate catalytic domains [56, 58]. Site-directed mutagenesis demonstrates that the two domains do not require each other for catalytic activity. If the corresponding histidine residues in each catalytic domain are mutated to alanine, to produce the H216A and H611A single mutants and the H216/611A double mutant, mutation of either H216 or H611 to alanine only slightly reduces HDAC activity, while simultaneous mutation of both sites abrogates this activity completely. Furthermore, a truncation mutant of HDAC6 containing the N-terminal 460 aa, and therefore only the first catalytic domain, is still catalytically active. Therefore, both catalytic domains of HDAC6 are fully functional HDACs and contribute independently to the overall activity of the wild-type HDAC6 protein [56, 59–61]. HDAC6, like all other HDACs, is inhibited by Trichostatin A (TSA); however, HDAC6 is uniquely resistant to the potent HDAC inhibitors trapoxin-B and sodium butyrate (NaBut). These drugs were used to demonstrate both in vitro and in vivo that HDAC6 is a deacetylase for nonhistone proteins, like α-tubulin [51, 62].

One of the functions of HDAC6 is as an Hsp90 deacetylase. Inactivation of HDAC6 results in the accumulation of acetylated Hsp90, which no longer forms a stable complex with steroid receptors such as the glucocorticoid receptor (GR) [52, 63]. In HDAC6-deficient cells, Hsp90-dependent maturation of the GR is compromised, resulting in a GR that is defective in ligand binding, nuclear translocation, and transcriptional activation. These findings identified Hsp90 as a target of HDAC6 and suggested reversible acetylation as a unique mechanism by which the Hsp90 chaperone complex activity is regulated [39, 52, 64–66]. Because AR forms chaperone complexes with Hsp90, and HDAC6 is required for stabilization of AR protein [67, 68], these findings strongly implicate that, at least in part HDAC6-mediated acetylation/deacetylation of Hsp90 is a potential mechanism regulating steroid

hormone signaling, including AR signaling. Thus, we investigated whether HDAC6 could modulate AR signaling pathways through an Hsp90 acetylation-independent way.

HDAC6 is Required for Androgen-Independent AR Nuclear Localization in Castration-Resistant Prostate Cancer Cells

As discussed above, ligand-independent nuclear localization of both endogenous AR and transfected GFP-AR in CRPC C4-2 cells can be prevented when hsp90 is inhibited [36]. Studies also show that ligand-independent nuclear localization of AR requires HDAC6 deacetylase activity [69]. In this study, CRPC C4-2 cells were transfected with GFP-AR. Then the transfected cells were treated with the pan-HDAC inhibitor, TSA, or with NaBut, which inhibits all HDACs except HDAC6 [70]. It has been verified that GFP tagging does not affect the function, subcellular localization, or stability of the AR protein [71–73]. Without ligand, GFP-AR in C4-2 cells is predominately nuclear, in contrast to the predominant cytoplasmic localization of GFP-AR in androgen-sensitive LNCaP cells. As a control, GFP alone is evenly distributed in both LNCaP and C4-2 cells. In C4-2 cells, TSA treatment produces a shift of GFP-AR to the cytoplasm, whereas NaBut treatment does not. Western blots showed that TSA slightly enhances GFP-AR expression, indicating that the redistribution of GFP-AR is not caused by selective degradation of nuclear GFP-AR. Thus, inhibition of HDAC6 deacetylase activity by TSA prevented the ligand-independent nuclear localization of GFP-AR in castration-resistant C4-2 cells [69]. Since nuclear localization of AR is a prerequisite of its activation, inhibition of AR nuclear localization consequently should inhibit AR transcriptional activity.

HDAC6 Regulates AR Hypersensitivity

HDAC6 appears to play a critical role in regulating AR sensitivity to androgens, particularly the hypersensitivity in CRPC [69]. In C4-2 cells, HDAC6 knockdown inhibits PSA mRNA expression in the absence or presence of DHT. Also, HDAC6 knockdown reduces cell proliferation in both ligand-free and complete medium. Interestingly, DHT still induces PSA mRNA in C4-2 cells with knockdown of HDAC6, indicating that AR remains androgen responsive. Consistent with this, there are studies suggesting that AR activation, as reflected by PSA expression in CRPC, is androgen independent [5]. Hypersensitization of AR to castrate levels of androgens in prostate cancer is thought to be a major mechanism leading to castration resistance [27]. The inhibition of PSA expression in C4-2 cells by knockdown of HDAC6 cultured in charcoal-stripped medium indicates that HDAC6 contributes to AR hypersensitivity.

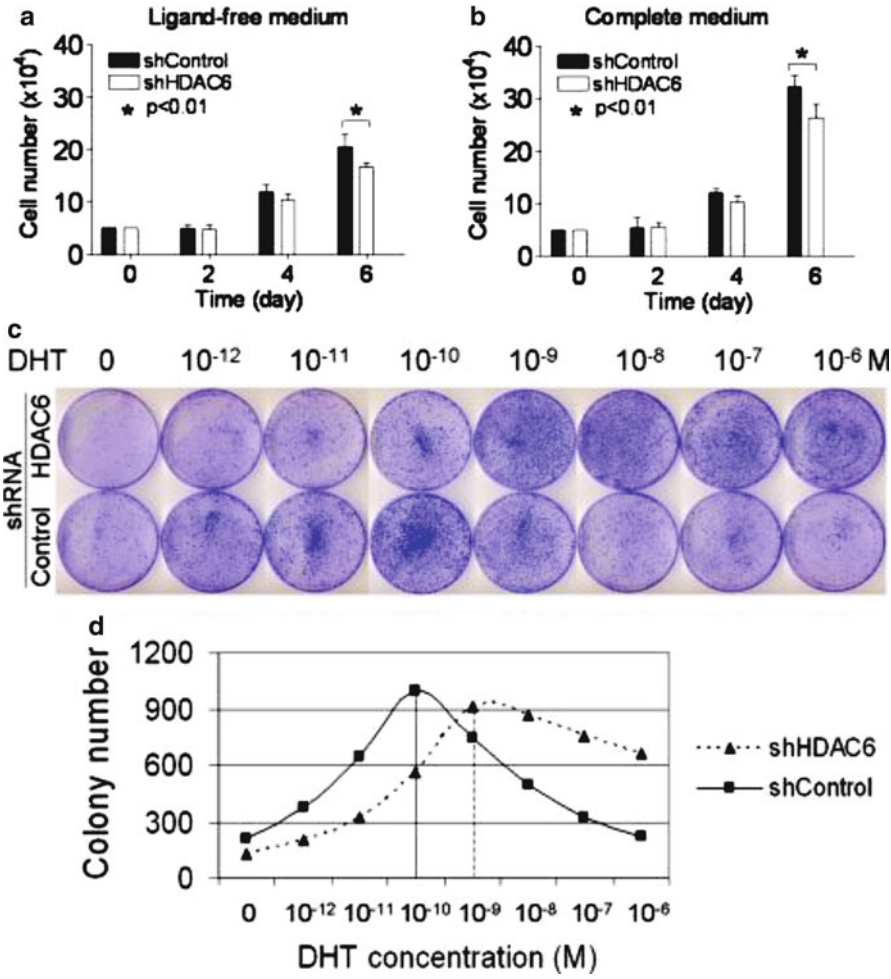

Fig. 16.1 Effect of HDAC6 knockdown on PCa cell growth. Control and HDAC6-knockdown C4-2 cells were cultured in ligand-free (**a**) or complete (**b**) medium with an initial 50,000 cells per well on six-well plates in triplicate. The cell number was counted at the indicated time points. The results presented here are representative of three independent experiments. *Error bars* represent ± sd. *, A *P* value <0.01 was generated using an unpaired *t* test in GraphPad Prism. (**c**) Control and HDAC6-knockdown C4-2 cells were seeded at 10,000 cells per 10-cm dish in triplicate. After 2 weeks in T-medium without or with the indicated concentrations of DHT, cell colonies were stained with crystal violet and colony numbers counted. The colony images are representative of three independent experiments. (**d**) Quantitative analysis of colony formation assay. The colony numbers represent means. [Adapted from Ai et al. [69]. ©2009, The Endocrine Society.]

Given that androgens stimulate cell proliferation and survival in prostate cancer cells through AR [74, 75], it is important to evaluate how inhibition of HDAC6 affects prostate cancer cell proliferation. Indeed, our studies [69] showed that HDAC6 knockdown slightly inhibited C4-2 cell proliferation (Fig. 16.1a, b).

However, knockdown of HDAC6 did not result in cell death in C4-2 cells. These data argue that HDAC6 inhibition causes little or no cytotoxicity. In addition, the effect of HDAC6 knockdown on colony formation in C4-2 cells was also determined at various androgen concentrations. C4-2 cells exhibited a bell-shaped growth response to increasing DHT concentrations, which is similar to the dose response of androgen-induced LNCaP growth [76]. C4-2 cells with HDAC6 knockdown developed visibly smaller colonies than C4-2 cells treated with control shRNA. Moreover, HDAC6 knockdown caused a shift in the dose response to DHT: whereas 10^{-10} m DHT stimulated the maximum colony formation in the control group, HDAC6-knockdown cells required 10^{-9} m DHT (Fig. 16.1c, d). The shift in the dose–response curve demonstrates that HDAC6 contributes to AR hypersensitivity [69].

HDAC6 Knockdown Inhibits Growth of Castration-Resistant C4-2 Xenograft Prostate Tumors

We [69] evaluated the effect of HDAC6 in vivo *using* human prostate xenograft tumors in nude mice and demonstrated that both HDAC6 knockdowns and controls of C4-2 cells generated tumors in six of six (100 %) intact mice. However, 6 weeks after injection, the mean volume of shRNA control C4-2 tumors in intact mice was statistically and significantly greater than that of C4-2 tumors with stable knockdown of HDAC6 by shRNA, supporting a role for HDAC6 in androgen-dependent prostate xenograft tumor growth. Knockdown of HDAC6 in C4-2 xenograft tumors resulted in a marked decrease in PSA expression while having little or no change in AR protein levels. This indicates that HDAC6 knockdown inhibits AR transcriptional activity, even in intact male mice. No tumor was formed in castrated nude mice injected with C4-2 cells following knockdown of HDAC6; whereas C4-2 cells treated with control shRNA formed tumors in two of six (33 %) castrated mice. These findings suggest that HDAC6 knockdown inhibits the establishment of castration-resistant prostate xenograft tumors in vivo.

Potential Mechanisms of HDAC6 in the Regulation of AR Signaling

Acetylation of Hsp90

AR forms complexes with chaperone Hsp90 [68]. Hsp90 functions via facilitating the structural maturation and complex formation of client proteins, including steroid hormone receptors and selected kinases [52]. HDAC6 regulates the activity of Hsp90. Inactivation or knockdown of HDAC6 leads to Hsp90 hyperacetylation, its

dissociation from cochaperone p23, and loss of chaperone activity. Loss of Hsp90 activity is likely to prevent AR maturation because Hsp90 is required for the maturation of the glucocorticoid receptor (GR), affecting ligand binding, nuclear translocation, and transcriptional activation [52, 63]. Knockdown of HDAC6 in prostate cancer C4-2 cells induces the hyperacetylation of Hsp90, impaired ligand-independent nuclear localization of endogenous AR, inhibited prostate-specific antigen (PSA) expression, and cell growth in the absence or presence of DHT [69]. Similar results were also observed in MEF cells [77].

The acetylation state of Hsp90 K294 is critical for its chaperone function [78]. This is consistent with our results that defect of GFP-AR nuclear translocation caused by HDAC6 deficiency is mediated mainly through acetylation of Hsp90. In the experiment, Hsp90 K294 mutants were used to determine whether acetylation/deacetylation of Hsp90 mediates HDAC6 regulation of AR nuclear localization. Expression of a wild-type or an acetylation-mimic Hsp90 mutant (Hsp90K294Q) in HDAC6-null MEF cells slightly increased the percentage of nuclear GFP-AR, whereas a deacetylation-mimic Hsp90 mutant (Hsp90K294R) markedly restored GFP-AR nuclear localization to levels seen with HDAC6 re-expression [69]. These findings strongly implicate HDAC6-mediated acetylation/deacetylation of Hsp90 as a potential mechanism regulating AR signaling.

Not only can inhibition of HDAC6 disrupt AR signaling by acetylating Hsp90. HDAC6 is also required for stabilization of AR protein. AR is normally stabilized by the chaperone activity of the Hsp90. Inhibition of HADC6 by soy isoflavone genistein or Sulforaphane downregulates AR in androgen-dependent prostate cancer cell lines such as LNCaP [67, 79]. Increased ubiquitination of AR after genistein treatment is attributed to decreased Hsp90 chaperone activity as assessed by its increased functionally inactive acetylated form. Consistent with this result, HDAC6 is inhibited by the antiestrogenic activity of genistein. These observations suggest a novel mechanism of AR protein down-regulation by genistein through inhibition of HDAC6-Hsp90 cochaperone function [67]. This mechanism is different from through inhibition of AR signaling while without changing AR protein stability.

AR Protein Stability

Given the centrality of AR in prostate cancer and the role of the cytoplasmic HDAC6 protein on activation of HSP90, which leads to AR protein stabilization, a study tested the hypothesis that sulforaphane treatment of prostate cancer cells would interfere with HDAC6 function and consequently lead to reduced levels of AR protein and attenuated AR signaling [52, 65, 80]. Sulforaphane treatment enhances HSP90 acetylation through HDAC6 inactivation, which leads to disruption of AR binding to HSP90, eventual AR degradation, and reduced expression of AR target genes [79]. As opposed to other compounds with HDAC inhibitory function, sulforaphane treatment leads to reduced AR binding to its target gene AREs [79]. Although sulforaphane may inhibit HDAC6 deacetylation of its tubulin substrate in

cell-free assays, much of the observed effect in prostate cancer cells may be due to degradation of HDAC6 protein after sulforaphane treatment [79]. Taken together, these studies suggest that inhibition of prostate cancer cell growth by HDAC inhibitors, including compounds such as sulforaphane with effects on HDAC6, is at least partially mediated through destabilizing AR protein levels.

Other Potential Pathways

A growing body of evidence has established the extensive cross talk between AR signaling and other signaling pathways such as growth factor, liver X receptor, ErbB2, beta-catenin, c-jun, mTOR, TGF-beta, Wnt, MAPK, TNFa, and IL-6. [81, 82]. As a cytosolic enzyme, HDAC6 catalyzes deacetylation of multiple nonhistone proteins in addition to Hsp90, like tubulin, F-actin-binding protein cortactin, beta-catenin, Ku70, etc. [61, 83, 84]. Identification of these and other proteins should be very helpful for further elucidating the mechanisms of HDAC6 regulation of AR signaling in prostate cancer.

Targeting HDAC6 for Prostate Cancer Treatment

To determine if a gene of interest is a feasible therapeutic target for a certain type of cancer, it is important to clarify its expression profile in different normal tissues and tissues of different cancer types. Northern blot analysis reveals that human HDAC6 is present as a 5-kb transcript and has the highest expression levels in heart, liver, kidney, and pancreas of normal human tissues [56]. The differences in tissue expression may reflect a tissue specific function of these enzymes. It has been established that HDACs are upregulated in most human cancers [85].

Thus far there are only limited studies on HDAC6 expression profile in prostate cancer. A study [86] of HDACs expression in human prostate cancer revealed distinct class I HDAC profiles between epithelial and stromal cells. In this study, HDAC6 transcripts were determined by quantitative RT-PCR in various prostate cancer cell line LNCaP, PC3, and DU-145 and 16 prostate cancer/normal tissue pairs. HDAC6 transcripts were detected in all the cell lines and tissues. HDAC6 mRNA levels among the three cell lines are fairly similar with only slight variability. The tumor/normal HDAC6 mRNA ratios were variable, but none of the ratios was greater than 1.25. There is no study regarding the relative protein levels of HDAC6 in human prostate cancer tissues and their corresponding normal counterpart. Further detailed studies are necessary to examine the expression profile of HDAC6 in prostate cancer. It will also be important to test whether HDAC6 activity is different between prostate cancer cells and normal cells. These studies will help determine whether targeting HDAC6 has selectivity for killing prostate cancer cells.

HDAC6 plays a pivotal role in cancer. HDAC6 is required for oncogenic cell transformation and modulates cell migration, invasion, motility, and radiation sensitivity [53, 87–90]. Inhibition of HDAC6 delays cell cycle in transformed cells [91]. AR signaling is regulated by HDAC6, at least in part through modulating acetylation of Hsp90 [69]. The diverse functions of HDAC6 in cancers suggest that it is a potential therapeutic target for the treatment of cancers, including prostate cancer. In general, HDAC inhibitors would be expected to retard tumor growth or cause cell death via modulating various signaling pathways [92, 93]. Indeed, HDAC inhibitors are an emerging class of anticancer agents with promising preclinical antitumor activity in both in vitro and in vivo studies in a wide range of cancers. Based on these preclinical findings, more HDAC inhibitors have undergone a rapid phase of clinical development. Some of them have entered Phase I–III clinical trials, both as single agents and in combination with other therapies, like Suberoylanilide hydroxamic acid (SAHA), varinostat, MS-27-275, BML-210, M344, and CI-994 [94, 95].

Clinical trials with HDAC inhibitors in prostate cancer thus far are limited. HDAC inhibitor therapy in prostate cancer appears rational including a recent report, which showed that class I HDACs are essential coactivators of AR [96]. The authors found that two widely used HDAC inhibitors, vorinostat and LBH589, block transcriptional activation of many AR targets, including *TMPRSS2-ERG*. This effect was recapitulated by siRNA to HDAC1, and, to a lesser extent, by siRNA to HDAC3 [96]. Although certain HDAC inhibitors can reduce AR protein levels in the cell, transcriptional suppression of AR targets in this report was independent of AR protein levels. Further, the authors showed that certain HDAC inhibitors do not block AR recruitment to its targets, rather, they suppress AR target genes activation by blocking the recruitment of AR coactivators and RNA polymerase II [96]. More importantly, to mimic CRPC, these investigators utilized a prostate cancer cell line LNCaP-AR, generated by over-expression of AR. The cells exhibit high basal expression of AR targets in the absence of androgens, can grow in castrate mice and are resistant to anti-androgen. They found that HDAC inhibitors suppress AR target expression in this cell line as well [96]. These findings have implications for the treatment of CRPC, and also underscore the need for more specific and less toxic HDAC inhibitors in the treatment of this disease.

Not only can HDAC inhibitors disrupt AR signaling, they can also suppress AR expression by reducing AR protein levels in the cell [80, 96–98]. HDAC inhibition may be achievable through dietary compounds such as sulforaphane, which is derived from cruciferous vegetables, whose high consumption is associated with lower prostate cancer risk, although the mechanisms for this remains unclear [99–103]. HDAC inhibitors, such as sulforaphane with effects on HDAC6, inhibit prostate cancer cell growth, which is at least partially explained by its effects on AR signaling. Given the findings that inhibition of HDAC6 by shRNA prevents androgen-independent nuclear localization and inhibits castration-resistant xenograft prostate tumor growth, it would be promising to target HDAC6 for treatment of both androgen dependent and CRPC [69].

Conclusions

It has long been recognized that AR signaling has a central role in all stages of prostate cancer, particularly in CRPC. Thus, novel therapeutic approaches targeting the regulation of AR are promising directions. HDAC6 modulates AR signaling by acetylation of Hsp90, a critical chaperone protein for AR conformational maturity, intracellular trafficking, and activation. It also regulates AR expression by reducing AR protein stability. In addition, HDAC6 exhibits effects on oncogenic cell transformation, cell cycle, migration, invasion, cellular stress response, which may or may not involve AR signaling. These findings suggest that HDAC6 should be a promising therapeutic target for not only androgen dependent but also CRPC. Regardless of these research advancements, it will be important to further understand the mechanisms of HDAC6 regulation of AR signaling in prostate cancer, which may promote the translation of HDAC6 from research into the clinic.

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Chapter 17
Beyond the Cell Cycle: Implications of D-type Cyclin Deregulation in Prostate Cancer

Michael A. Augello, William F. Ostrander Jr., and Karen E. Knudsen

Abstract D-type cyclins hold well-known roles in promoting cell cycle progression, mediated largely through activation of CDK4/6 kinase complexes. While decades of research from both in vitro and in vivo models confirmed these kinase-dependent roles in cell cycle initiation, only recently have additional, kinase-independent functions of the D-cyclin family been uncovered. Critical analyses of the Cyclin D1 interactome uncovered a large network of transcriptional regulators in complex with and modulated by Cyclin D1, among them the superfamily of steroid nuclear receptors. As such, the D-cyclin family of proteins have been implicated as critical regulators of cancer progression in many hormone-dependent diseases. In the context of prostate cancer, D-cyclin have been demonstrated to directly influence the activity of the androgen receptor (AR), whose activity is critical for both cellular growth and survival. Here, the roles of the D-type cyclin family in prostate cancer will be discussed in relation to both their "classical" kinase-dependent as well as kinase-independent functions, with specific focus on their influences on AR-driven tumor phenotypes.

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Abbreviations

Aa	Amino acid
AR	Androgen receptor
CDK	Cyclin-dependent kinase
CRPC	Castration resistant prostate cancer
DBD	DNA binding domain
DHT	Dihydrotestosterone
ER	Estrogen receptor
HDAC	Histone deacetylase
LBD	Ligand binding domain
mTOR	Mammalian target of rapamycin
PCa	Prostate cancer
PPARγ	Peroxisome proliferator-activated receptor gamma
PSA	Prostate-specific antigen
RB	Retinoblastoma tumor suppressor
RD	Repressor domain
TR	Thyroid-hormone receptor

Clinical Prostate Cancer Incidence

Prostate cancer (PCa) represents a major health concern in the USA and remains the most commonly diagnosed non-cutaneous malignancy in American men [1]. It is predicted that nearly 200,000 men will develop the disease annually with over 32,000 of these patients succumbing to death related to PCa [2]. Given the prevalence of this disease, current efforts are focused on early detection of primary lesions as this affords patients the greatest chance for successful therapeutic intervention. At time of diagnosis, if tumors are localized to the prostate, patients are effectively treated by either radical prostatectomy or radiation therapy. Unfortunately, development of metastatic lesions is common and requires systemic therapy to treat non-organ confined disease [3–5]. While initially effective (as evidenced by tumor regression), tumors refractory to treatment modalities ultimately arise, and it is this stage of the disease that is responsible for the majority of PCa-related deaths. As a result, intense efforts are focused on indentifying new avenues of therapeutic intervention that could prove effective at curtailing tumor growth and progression to this stage.

Work by Huggins and Hodges in the early part of the twentieth century led to the development of the first effective treatment for PCa, androgen deprivation therapy [6–9]. Incredibly, this continues to provide the greatest benefit to patients with disseminated disease by diminishing tumor burden and growth [3, 4, 10]. At the cellular level, androgen action is manifested through activity of the androgen receptor (AR), wherein circulating androgens (testosterone or dihydrotestosterone-DHT)

bind to and activate AR; this results in the induction of transcriptional programs that promote tumor cell survival and initiate entry into the cell cycle [10–12]. Deprivation of circulating androgens and/or introduction of AR antagonists limits the activity of AR and results in cytostasis or cell death [13], underscoring the importance of the AR signaling axis in PCa progression. Tumor regression is confirmed clinically though measured declines of serum levels of prostate-specific antigen (PSA), which is a direct target of AR and an effective metric of both AR activity and tumor growth [3, 14]. Unfortunately, within a relatively short time frame, a large majority of tumors develop resistance to AR-directed therapeutics, evolving mechanisms to reactivate AR signaling despite continued therapeutic intervention [3, 10, 15]. This stage is termed castration resistant prostate cancer (CRPC) and represents the most lethal form of the disease. Despite the identification of a multitude of mechanisms through which tumors promote aberrant AR activity [3, 4], few effective treatment options have been developed to curtail tumor growth at this stage. Indeed, even cytotoxic chemotherapeutics (e.g., taxanes, which show clinical benefit in many solid tumors) provide a minimal survival advantage to patients with CRPC [16]. Consequently, there is a critical need to identify clinical targets that can limit tumor growth.

Mechanisms of Cell Cycle Progression

Under non-mitogenic conditions, cells are maintained in G0 of the cell cycle through action of the retinoblastoma tumor suppressor (RB), which remains hypophosphorylated and bound to the E2F family of transcription factors. In this state, RB functions to recruit corepressors (e.g., SMRT and NCOR) to relevant E2F target gene loci, thereby attenuating E2F transcriptional output required for entry into S-phase [17]. Consequently, effective initiation and progression through the cell cycle requires ordered activation of key cyclin–CDK complexes (Fig. 17.1a), which phosphorylate and functionally inactive RB. Upon mitogenic signaling, levels of D-type cyclins accumulate, which bind and activate Cyclin-Dependent Kinases 4 and 6 (CDK4/6). Parallel pathways activate Cyclin E-CDK2 complexes, and the resultant surge in CDK activity inactivates RB though the ordered phosphorylation of key serine and threonine residues ultimately displacing RB from E2F-family transcription factors [18]. Activated E2F commits the cell to the cell cycle and promotes expression of genes required to progress through S-phase, including Cyclin A [19]. The subsequent rise in Cyclin A levels facilitates assembly with CDK2 and/or CDK1, which fosters completion of S-phase and entry into G2 [25, 26]. Thereafter, enhanced expression of Cyclin B1 promotes the formation of CDK1/Cyclin B1 complexes, which drive the cell through G2 and promote commitment to mitosis [26].

CDKs underpin RB pathway control, and their activity is, therefore, tightly governed through multiple limiting mechanisms often associated with cyclin–CDK complex inactivation. These include: regulation of cyclin binding; CDK

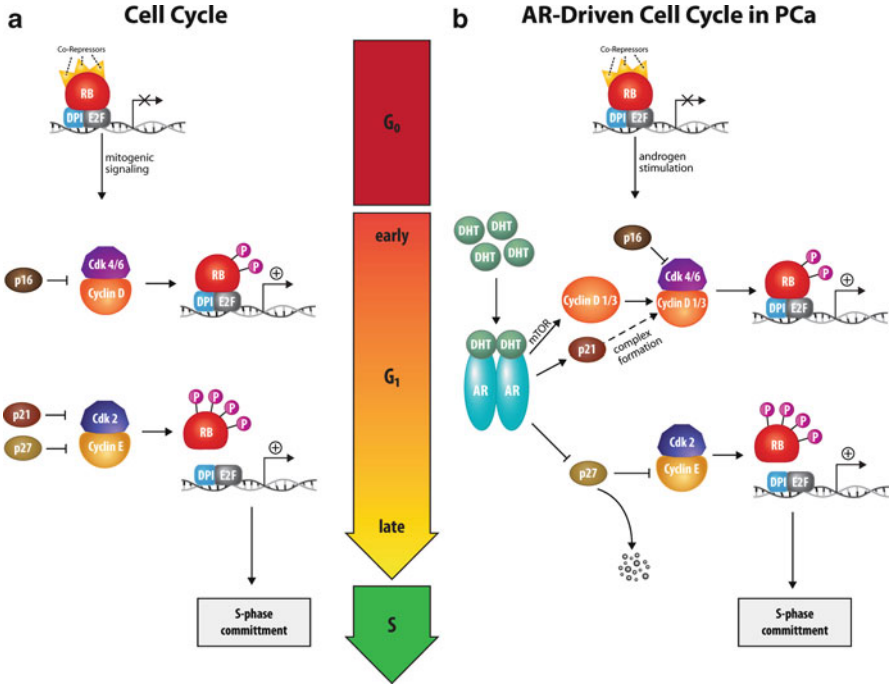

Fig. 17.1 *Mitogenic and androgen receptor driven cell cycle initiation* (**a**) RB recruitment of transcriptional corepressors to E2F loci causes inhibition of E2F target gene transcription, sequestering the cell in G0 [17]. Mitogenic stimulation triggers entry into early G1, wherein RB hyperphosphorylation by Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes results in derepression of E2F transcription of S-phase-promoting genes [18], thereby fostering S-phase commitment and cell cycle progression [19]. Efficient progression through the cell cycle is maintained via ordered activation of CDK/Cyclin complexes, whereby the CDK inhibitors p16 and p21/p27 serve to tightly regulate progression through G1 into S-phase (manifested through inhibition of Cyclin D/CDK4/6 and Cyclin E/CDK2, respectively) [20]. (**b**) Upon androgen stimulation in PCa cells, indirect transcriptional programs governed by AR promote mToR-dependent accumulation of Cyclins D1/3 and formation of active Cyclin D-CDK4/6 complexes [21]; these function to both phosphorylate RB and shunt p21 away from Cyclin E/CDK2 complexes [22], whose activity is further enhanced by AR-mediated p27 degradation [23, 24]. Collectively, these pathways maximize the activity of Cyclin E/CDK2 complexes, functionally inactivating RB, which fosters activation of E2F transcription and cell cycle commitment

phosphorylation states (via phosphatases such as CDC2); and CDK inhibition via action of $p16^{Ink4A}$, $p21^{Cip1}$, and $p27^{Kip1}$ [20]. $p16^{Ink4A}$ is rapidly induced by antiproliferative signaling and binds the Cyclin D-CDK4/6 complex to inhibit its kinase activity against RB, whereas both $p21^{Cip1}$ and $p27^{Kip1}$ bind the Cyclin E-CDK2 complex and limit its kinase activity [20, 27]. In addition to its inhibitory role, $p21^{Cip1}$ is known to facilitate Cyclin D-CDK4/6 kinase activity via promoting formation, stabilization, and nuclear accumulation of the complex [27–29]. $p27^{Kip1}$ levels are

altered during cell cycle progression. In G0 and G1, $p27^{Kip1}$ levels are elevated and associated with cyclin/CDK complexes in an active state. As cell cycle progresses, $p27^{Kip1}$ is phosphorylated and inactivated, allowing Cyclin D-CDK4/6 to phosphorylate RB and shunt $p27^{Kip1}$ away from Cyclin E-CDK2, promoting $p27^{Kip1}$ degradation and RB hyperphosphorylation [22]. Together, these regulatory processes ensure ordered progression through the cell cycle.

AR Function and Cell Cycle Progression

AR is a ligand-dependent transcription factor belonging to the nuclear receptor superfamily and containing conserved DNA-binding and ligand-binding domains and a unique C-terminus [11, 30, 31]. Prior to activation, AR is sequestered in the cytoplasm through association with heat-shock proteins, which limits basal AR function. Upon ligand binding, AR is released from these inhibitory heat-shock proteins, thus facilitating homo-dimerization and rapid translocation to the nucleus. In the nucleus AR localizes to transcriptional regulatory elements on chromatin (promoters and enhancers) and elicits context specific transcriptional programs that govern a multitude of cellular processes [32]. Of specific interest are the AR-driven networks that promote cell cycle entry and subsequent tumor growth. Multiple direct and indirect AR-driven pathways have been identified that impinge upon cell cycle progression, most of which are critical for G1-S progression [33, 34]. In the absence of androgen, PCa cells arrest in G0 and CDK4/6 complexes remain inactive due to the limited expression of D-type cyclins and elevated levels of $p27^{Kip1}$ [21, 34]. Accordingly, androgen stimulation promotes mTOR-dependent accumulation of both Cyclin D1 and D3, which is sufficient to promote CDK4/6 kinase activity [21]. These events are independent of changes in D-cyclin transcript levels, suggestive of an indirect action of AR in Cyclin D accumulation [21, 35]. Activated complexes function to initiate cell cycle entry through inactivation of RB, a gatekeeper of S-phase entry, through coordinated phosphorylation of key serine and threonine residues [36]. Thus, AR acts to promote cell cycle entry through regulation of D-type cyclin protein levels (Fig. 17.1b).

AR governs additional parallel pathways that promote completion of G1 and transition to S-phase (Fig. 17.1b). Similar to CDK4/6, CDK2 complexes are largely inactive in the absence of androgen [23]. Interestingly, there is little evidence that Cyclin E levels (a binding partner of CDK2) are directly altered by androgen status [21, 23]. This suggests that ADT induced repression of CDK2 activity is independent of Cyclin E status. Instead, this effect is likely a result of the androgen-mediated regulation of the CDK2 inhibitor $p27^{Kip1}$, which is rapidly degraded upon androgen stimulation [23, 24]. Though the mechanisms through which AR regulates $p27^{Kip1}$ are still under investigation, the resultant surge of CDK2 activity is thought to effectively inactivate RB, promote entry into S-phase, and commitment to the cell cycle. Thus, cell cycle initiation and progression is critically dependent on the function of AR signaling in PCa cells.

Transcriptional Functions of Cyclin D1

Cyclin D1 is a well-characterized regulator of the cell cycle whose ability to promote entry into S-phase contributes to the progression of many human cancers (e.g., breast cancer, gastric cancer) [20]. However, a wealth of evidence, from both in vitro and in vivo models, has uncovered a larger (kinase independent) role for Cyclin D1 in numerous biological processes including DNA damage repair and transcriptional regulation [20, 37–41]. Consonantly, in vivo proteomic–genomic screens from multiple tissue types found that an overwhelming majority of the Cyclin D1 interactome belongs to the superfamily of transcriptional regulators [37]. Studies aiming to uncover the underpinning consequences behind such associations demonstrated that Cyclin D1 associates with DNA-bound complexes, which directly regulate the transcription of many genes associated with development and differentiation [37]. For example, Cyclin D1 resides at the promoter of the gene *Notch1*, whose expression is required for proper development of the retina [37]. Upon loss of Cyclin D1, *Notch1* expression was dramatically reduced and resulted in hypoplastic morphology within retinal tissue [37]. Importantly, expression of a mutant allele of Cyclin D1 (deficient in its ability to activate CDK4/6 kinase activity) completely reversed the retino-hypoplastic phenotype exhibited by the *Ccnd1* D1 −/− mouse, underscoring the importance of Cyclin D1 transcriptional control in normal cell development [40]. It should be noted that the transcriptional attributes of Cyclin D1 are not limited to ocular tissues, as numerous families of transcription factors are found in direct association with Cyclin D1. Among the most prominent of these members are the superfamily of nuclear receptors including the estrogen receptor (ER), thyroid hormone receptor (TR), peroxisome proliferator-activated receptor gamma (PPARγ), and the androgen receptor (AR) [20, 42–47]. Unlike its role in cell cycle progression, Cyclin D1 acts in a cell type and cofactor-specific manner to regulate nuclear receptor transcriptional activity. In the context of PCa, Cyclin D1 directly regulates AR activity, and as will be discussed, has major implications for the development and progression of PCa.

AR-Cyclin D Feedback

Cyclin D1

While it is well established that AR signaling impinges upon the cell cycle machinery through multiple mechanisms, a large body of evidence suggests that cell cycle components can feedback and directly influence AR activity. With regard to the cell cycle, the D-type cyclins are the best-characterized regulators of AR and largely function to dampen AR activity [13, 48–51]. In the context of PCa, Cyclin D1 acts as a modulator of transcription and cell cycle progression by repressing ligand-dependent AR activity [34, 51]. These functions are manifested

through direct binding of Cyclin D1 to the AR N-terminal LxxLF motif, which disrupts AR N-C terminal interactions that necessitate AR transactivation and direct DNA binding [52]. In addition, Cyclin D1 further acts to regulate AR activity through modulation of the local chromatin environment. In response to androgen, Cyclin D1 is recruited to sites of AR action and associates with histone deacetylaces (HDACs) [42], limiting open chromatin architecture required for transcriptional induction. Cyclin D1 achieves this regulation of AR activity via its central repressor domain, RD (amino acids 142–254 of Cyclin D1), which contains an FxxLF motif both necessary and sufficient for binding to AR and repressing ligand-dependent activation without altering AR levels [42, 47, 51]. Introduction of the RD alone is sufficient to diminish not only expression of AR target genes but also cell growth and viability, illustrating the importance of Cyclin D1 transcriptional functions on cellular outcomes [51]. Indeed, independent gene expression analyses in models of Cyclin D1 upregulation confirmed the repressive function of Cyclin D1 on many direct AR target genes (*KLK2*, *KLK3*, and *TMPRSS2*), further supporting the concept of Cyclin D1 as a potent regulator of AR activity [53]. Importantly, these analyses also uncovered subsets of genes that are similarly induced or repressed by both Cyclin D1 and AR [53]. Though there is little evidence that AR directly regulates these gene clusters, such data imply that the ability of Cyclin D1 to regulate AR activity is highly complex and locus specific. As such, further (genome wide) analyses are required to fully understand the role of Cyclin D1 on tumor-associated AR function.

Considering the above findings, it is not surprising that deregulation of Cyclin D1 expression is a common event in early stage disease. As will be discussed, clinical samples illustrate multiple mechanisms through which tumors limit Cyclin D1 function including: downregulation of protein expression [35, 54], cytoplasmic mislocalization [54], and most recently alternative splicing [35]. While this alternative splicing event is not readily detected in normal tissue, a large number of primary prostate cancers are enriched for the product of this event, a protein dubbed Cyclin D1b [35, 55]. The Cyclin D1b transcript (*transcript b*) arises as a failure to splice at the exon 4/intron 4 boundary of Cyclin D1 pre-mRNA [49] (Fig. 17.2). This results in the incorporation of intron 4 coding sequences and the introduction of an early stop codon. Consequently, Cyclin D1b protein lacks all terminal exon 5 coding sequences and harbors a unique 33 amino acid C-terminus (encoded by intron 4) (Fig. 17.2) [49]. While the biological events that promote *transcript b* expression are still under investigation, several oncogenic and non-oncogenic factors have been identified which impinge upon *transcript b* production in PCa [56, 57]. For example, the splicing factor SRSF1 (also known as SF2 and ASF1) is a known oncogene of PCa relevance, which can promote alternative splicing and production of *transcript b* (and Cyclin D1b) in vitro [57]. In silico analyses revealed a putative SRSF1 binding site at the exon4/intron 4 boundary of the *CCND1* pre-mRNA, which harbors a common polymorphism at a critical splice acceptor site, G/A870 [57]. While the presence of the A allele results in higher *transcript b* production than the G allele in normal cells, this advantage is lost in transformed PCa cells [57]. Such results are likely

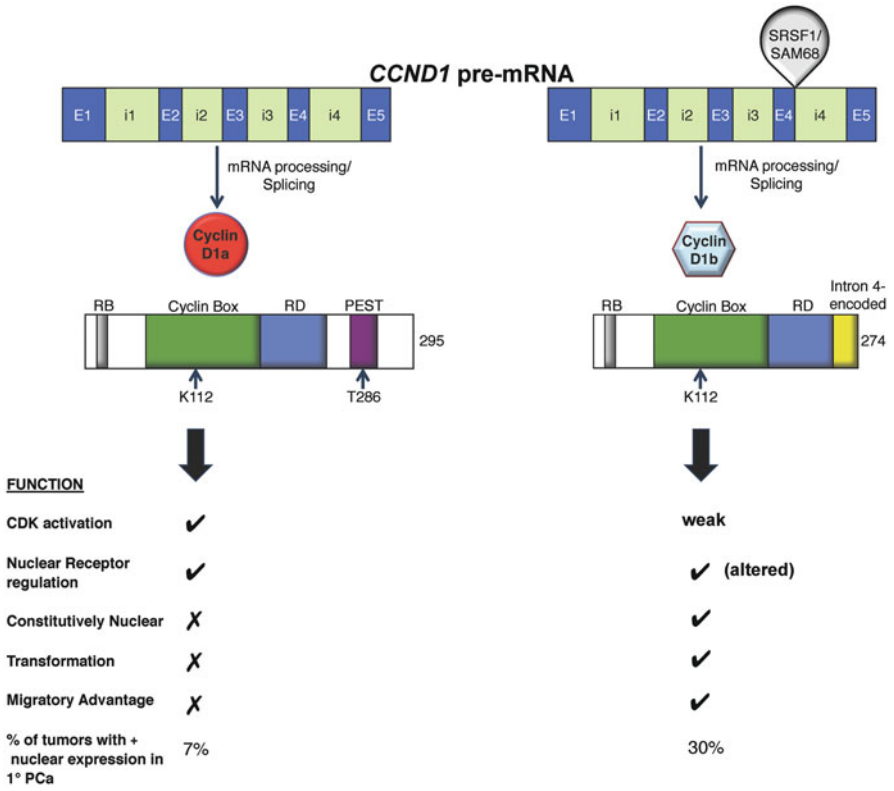

Fig. 17.2 *Mechanism and function of Cyclin D1 alternative splicing*. The Cyclin D1 full-length transcript consists of 5 exons encoding functional domains necessary for its biochemical functions and stability. As a consequence of disease progression (or enhanced expression of critical splicing factors such as SRSF1), the *CCND1* transcript is alternatively spliced to include intron 4 sequences. This results in the introduction of an early stop codon culminating in the exclusion of exon 5 encoding sequences and production of a novel C-terminus. This variant, dubbed Cyclin D1b, while maintaining residues required for CDK4 binding/activation (K112), is missing key regulatory elements necessary for its transcriptional functions (terminal sequences of the Repressor Domain-RD) as well as stability (T286). Consequently, Cyclin D1b has been demonstrated to harbor divergent functions from those of full length Cyclin D1 (Cyclin D1a), associated with enhanced oncogenic potential of this variant both in vitro and in vivo [35, 49, 55–59]

attributed to the induction of SRSF2 expression, which binds more readily to transcripts containing the G allele, resulting in a surge of *transcript b* (and resultant Cyclin D1b) expression in PCa [57]. Additional (non-oncogenic) splicing factors whose induction is also associated with *transcript b* have been identified (e.g., SAM68), but the molecular mechanisms that underlie their function are still unclear [56]. Thus, a primary mechanism by which Cyclin D1 function is abrogated in PCa is manifested through alternative splicing events, which results in production of Cyclin D1b, and vastly disparate biochemical outcomes.

While the transcriptional and cellular functions of full length Cyclin D1 (Cyclin D1a) have been studied in detail in many systems, the biochemical consequences of Cyclin D1b induction have only recently been explored [49]. Importantly, despite a high degree of homology, there are few functional similarities between the two Cyclin D1 isoforms (Fig. 17.2). First, while Cyclin D1a is a potent inducer of CDK4/6 activity, Cyclin D1b is limited in this ability (despite maintaining CDK4/6 binding capacity) [49]. Second, Cyclin D1b lacks critical exon 5 encoding sequences (namely T286) necessary for efficient nuclear export and degradation, resulting in constitutively nuclear localization [58]. Third, expression of Cyclin D1b (but not Cyclin D1a) is sufficient to promote colony formation and migration in several models of cancer progression, demonstrating the enhanced oncogenic potential of this splice variant [58, 59]. Finally, while maintaining the ability to efficiently bind to nuclear receptors (including AR), Cyclin D1b functions in an altered capacity to regulate their activity (disparate from that of full length Cyclin D1a). For example, induction of Cyclin D1b expression is unable to negatively regulate AR activation and resultant AR transcriptional activity [55]. Accordingly, cells harboring Cyclin D1b upregulation demonstrate a growth advantage in androgen proficient conditions [55], indicating that Cyclin D1b likely manipulates the AR signaling axis to promote aggressive tumor phenotypes. Critical next steps will aim to define the consequences of Cyclin D1b expression on the AR cistrome and transcriptome to identify AR/Cyclin D1b-driven targets that underlie the oncogenic properties of Cyclin D1b in PCa.

Cyclins D2 and D3

While Cyclins D2 and D3 share a high degree of homology with that of Cyclin D1a [20], there are only a limited number of studies which have assessed their expression and function in cancer associated phenotypes. In the context of PCa, biochemical functions seem to be maintained whereby both Cyclin D2 and D3 retain the capacity to bind and activate CDK4/6, resulting in RB phosphorylation and entry into S-phase [20]. Moreover, evidence also suggests that the transcriptional functions of Cyclin D2 and D3 are similar to that of Cyclin D1, exerting ligand-dependent, kinase-independent regulation over AR activity [48, 60]. While both Cyclin D2 and Cyclin D3 possess AR-binding capacity, there is little evidence that Cyclin D2 is expressed to any appreciable degree in PCa [48, 60, 61]. Inversely, Cyclin D3 levels, which are abundant in PCa cells, repress ligand-dependent AR activity via CDK-independent, RD-mediated mechanisms that closely mirror those of Cyclin D1a [48]. Likewise, Cyclin D3 over-expression reduced AR recruitment to the *KLK3* (PSA) locus in response to DHT and resulted in a proliferative reduction in PCa cell lines [48]. Unlike Cyclin D1a however, Cyclin D3 possesses kinase-dependent mechanisms that can influence AR function. CDK11^{p58}/Cyclin D3 complexes function to suppress AR activity via direct phosphorylation of AR at Ser-308, resulting in decreased transcriptional output and cell growth in vitro [62]. Unfortunately, the

consequences of Cyclin D3 induction on AR activity and tumor growth in vivo have not been defined, and further investigation is required to elucidate the role of Cyclin D3 in PCa progression.

Cyclin D1-DNA Damage Repair

A wealth of evidence is suggestive that a major function of Cyclin D1 in PCa cells is independent of its role in cell cycle progression. This concept is supported by recent in vivo proteomic/genomic screens identifying cell cycle components as only a minor category of Cyclin D1 interacting protein families. Outside of transcriptional regulators, DNA damage-associated proteins emerged as the second largest family that demonstrated Cyclin D1 association [37, 38, 63]. Prior to these studies, Cyclin D1 had been suggested to play a role in either fostering or inhibiting DNA repair post genotoxic insult, depending on the cellular context [39, 64–69]. In hormone-driven diseases (e.g., breast cancer) the former appears to be true; the functional interactions between Cyclin D1 and DNA damage repair machinery were validated, wherein a direct interaction was found between Cyclin D1a and RAD51, which was greatly enhanced post genotoxic insult [38]. Furthermore, Cyclin D1 was found to co-localize with RAD51 at sites of double stranded breaks, and depletion of Cyclin D1 protein resulted in a dramatic reduction in RAD51 occupancy at sites of DNA damage as well as rates of DNA repair [38]. As Cyclin D1a has no known DNA binding motifs, it has been postulated that Cyclin D1 functions as a linker protein, tethering DNA repair proteins (like RAD51) to sites of DNA damage marked by damage sensing protein family members. Once such class of proteins, the BRCA family, localizes to damage foci early in strand repair [70], and facilitates the assembly of repair machinery. Importantly, BRCA2 has been implicated as a direct binding partner of Cyclin D1a [71], whose ability to localize to sites of DNA damage is unaffected by Cyclin D1a levels [38]. These data suggest a model wherein Cyclin D1 functions to recruit DNA repair machinery to sites of damage mediated though a tethering mechanism.

Though most of these studies have been conducted in models other than prostate, such findings have potential implications for both DNA repair and cell survival in PCa cells. Emerging evidence suggests that AR signaling acts to promote cell survival and DNA repair post genotoxic insult [72]. Given the knowledge that AR signaling initiates accumulation of Cyclin D protein levels, it is tempting to speculate that at least part of the AR-directed DNA damage repair program involves Cyclin D-mediated DNA damage repair. Future efforts examining isoform-specific Cyclin D interacting partners post-insult will help define the role of D-type cyclins in PCa and aid in the identification of novel biological targets which would enhance the efficacy of primary treatments.

Cyclin D1 and Cell Survival

Given the expanding, kinase-independent roles attributed to D-type cyclins, a concerted effort is being made to define and understand the Cyclin D1 interactome. While great strides have been made to date, current data suggest that Cyclin D1 has cell type specific binding partners. Indeed, such events could help explain the cell type specific function of Cyclin D1 in the promotion of tumor phenotypes. For example, in a prostate-specific background, Cyclin D1a expression promotes cell survival through inhibition of anoikis [73]. Loss of signaling from key membrane-bound integrins (e.g., *ITGB4*) activates the FOXO family of proteins (specifically FOXO3a), which initiates transcriptional programs that mediate cell death [73]. Importantly, this cell death response correlates with a decrease in Cyclin D1a levels and hyperactive signaling though the FOXO3a pathway [73]. In these models, induction of Cyclin D1a post-cell detachment promoted the association of Cyclin D1/FOXO3a complexes resulting in the degradation of FOXO3a protein, diminished downstream FOXO3a signaling, and enhanced cell survival in suspension [73]. These data suggest that kinase-independent Cyclin D1a interactions are critical for tumor-associated phenotypes, promoting cell survival and progression to metastatic disease. To date, the relative contributions of the other D-type cyclin isoforms towards such phenotypes are unknown. As Cyclin D1b and Cyclin D3 are the major Cyclin D isoforms expressed in primary PCa, it will be critical to determine if they retain similar functions to that of Cyclin D1a in the DNA damage repair pathway in this context.

Clinical Implications for D-Type Cyclins in PCa

Cyclin D1 in Primary PCa

Expression of Cyclin D1a has been extensively studied in clinical samples of human disease. Given the critical role that D-type cyclins play in nuclear receptor control, it is not surprising that Cyclin D1a expression is often altered as a function of disease development in hormone-driven cancers. As mentioned above, Cyclin D1a is often dysregulated in primary PCa, likely attributed to the inhibitory role of Cyclin D1a on AR signaling in this tumor type. While there is little evidence for genetic deletion of the *CCND1* gene in primary PCa [74, 75], a large fraction of tumors harbor diminished Cyclin D1a expression [35, 54, 76–78]. Indeed analysis of Cyclin D1a expression in a large cohort of primary PCa samples found that nearly 60 % of all tumors display low to no Cyclin D1a expression, correlating with increased AR activity (as determined by an increase in serum PSA levels) [35]. Furthermore, of those tumors that demonstrated Cyclin D1a positivity, independent analyses found

a significant fraction of total Cyclin D1a localized to the cytoplasm, disrupting the biochemical functions of Cyclin D1a on AR-driven tumor phenotypes [54]. Unfortunately, few studies to date have examined clinical samples to distinguish between the expression of the two Cyclin D1 isoforms. Currently, PCa remains one of the only malignancies where such distinctions have been made. Cyclin D1b was detected in nearly 30 % of all primary tumors, with little or no expression found in neighboring matched non-neoplastic tissue [35]. Such data indicate that, as a function of tumor progression, there is a switch in preferential expression of the Cyclin D1 isoforms from Cyclin D1a to Cyclin D1b. Further investigation found Cyclin D1b expression in primary tumors to be independent of the polymorphism at the exon 4/intron 4 splice junction (G/A870), likely due to the enhanced expression of the splicing factor SRSF1 in primary disease [35]. Matched clinical samples found increased Cyclin D1b production in tumors that expressed high levels of SRSF1, providing one potential mechanism through which the Cyclin D1 switch occurs in primary PCa [57]. Overall, while alterations of total Cyclin D1 expression in primary disease (loss of Cyclin D1a or induction of Cyclin D1b) did not correlate with markers of proliferation (Ki67), grade, stage, or time to biochemical failure, emerging evidence from samples of metastatic lesions suggests Cyclin D1 may contribute to metastatic progression [35, 79]. Thus, in primary PCa, there is a general shift in Cyclin D1 isoform expression from Cyclin D1a to Cyclin D1b, whose specific roles likely involve manipulation of AR-driven pathways that promote cancer progression. While the biochemical roles of these isoforms are relatively well defined in local disease, their contribution and relative expression patterns in lethal metastatic disease is still under investigation, and critical next steps will aim to discern the unique role of the Cyclin D1 isoforms in this stage.

Cyclin D2 and D3 in Primary PCa

While Cyclin D1 expression has been extensively studied in primary PCa, there is only limited clinical evidence examining either Cyclin D2 or D3 levels. Similar to what is observed in vitro, Cyclin D2 expression is extremely low or undetectable in clinical samples [61, 80, 81]. The underlying mechanisms behind the limited detection of Cyclin D2 were uncovered through examination of genome wide methylation analyses of PCa samples. In normal tissue, the *CCND2* promoter was commonly found methylated, an event associated with transcriptional repression [61, 80, 81]. Analysis of matched neoplastic tissue found that these methylation events occurred more frequently with disease progression, where higher methylation status correlated with higher Gleason grade and serum PSA [61]. Similarly, Cyclin D3 expression was found to be generally lower in neoplastic tissue, which inversely correlated with proliferative markers [48]. Thus, unlike many other solid epithelial tumors, primary prostate cancers develop mechanisms to limit the expression of the canonical D-type cyclins, inhibiting their biochemical functions involved in cell cycle progression and transcriptional control.

Conclusions and Future Directions

Initially named because of the oscillation in their expression as a function of cell cycle progression, the D-type cyclin family members are best known for their roles in CDK activation and cellular proliferation. Modeling of D-type cyclin deregulation in murine backgrounds confirmed the importance of these kinase-dependent functions, but (more notably) uncovered a larger kinase-independent role for D-type cyclins in transcriptional control and the DNA damage response [37, 38]. In the context of PCa, the canonical D-cyclins appear to function as direct transcriptional regulators of AR, thereby limiting the strength and duration of AR signaling required for enhanced proliferative capacity [34]. As AR function is critical for disease progression, a large majority of tumors develop mechanisms to limit Cyclin D expression, presumably as a means to derepress signaling via the AR axis. A common method through which such deregulation occurs is through alternative splicing of the *CCND1* transcript, resulting in the production of Cyclin D1b, a variant that is altered in its capacity to regulate AR [34, 35]. Consequently, Cyclin D1b expression is induced as a function of disease progression and represents the most common form of Cyclin D1 in primary PCa [34, 35]. Gains in our understanding of the clinical expression patterns of D-type cyclins not only demonstrate the unique role of this family of proteins in PCa but also raise several questions that should be addressed. *First, what are the consequences of Cyclin D1b induction on prostate-specific tumor phenotypes?* Cyclin D1b has been shown to have enhanced oncogenic potential (as compared to full length Cyclin D1a) in multiple models of cancer progression [49]. As this variant represents the most common D1-type cyclin in primary disease, and is induced specifically as a function of cancer progression, it will be critical to define the oncogenic potential of this variant to better understand the means through which D-cyclins contribute to cancer-associated phenotypes. *Second, how does D-type cyclin deregulation affect the DNA damage response in PCa?* A multitude of studies in several cancer types suggest conflicting roles for Cyclin D1a in facilitating DNA damage repair [39]. Most recently in breast cancer, Cyclin D1a was found to localize to sites of double strand breaks and recruit critical repair complexes that mediate efficient and timely repair [38]. As clinical evidence suggests that D-type cyclin expression is altered in primary PCa, it would be interesting to determine if Cyclin D1 facilitates the assembly of similar repair complexes post-genotoxic insult. Accordingly, the contribution of Cyclin D1b and Cyclin D3 in DNA repair mechanisms should be determined, as they comprise the bulk of D-type cyclin expression in primary disease. If similar functions are shared with Cyclin D1a, Cyclin D1b/D3 could serve as novel biomarkers of radioresistance in primary disease. *Third, what is the expression pattern of each of the D-type cyclins in CRPC?* While the expression of the D-cyclin family has been relatively well documented in localized disease, such expression profiles have yet to be examined in advanced PCa. Given the multifaceted roles attributed to each of the D-cyclin isoforms, it will be critical to define their function in CRPC development and progression, and determine if such biochemical attributes are conserved at this stage.

Finally what are the consequences of D-type cyclin deregulation on the AR cistrome in advanced disease? Given the potent role D-cyclins play in regulating AR occupancy on chromatin and the knowledge that the AR cistrome changes as a function of disease progression, it will be important to define how changes in D-cyclin expression affect AR occupancy on chromatin (in CRPC). While several factors have been identified which affect the AR cistrome and contribute to AR-specific CRPC programs (e.g., FOXA1 and GATA2) [82–86], little is known as to how altered D-cyclin expression contributes to such programs. Uncovering such pathways will help to define the functions of the D-cyclin family in the promotion of lethal tumor phenotypes during the development of CRPC. Thus, functions of the family of D-cyclins have expanded well beyond their initially characterized role in cell cycle progression, regulating numerous cellular processes required for both tumor growth and progression. Future efforts should further characterize these functions, beyond their namesake, in hopes of identifying new clinical targets to exploit for therapeutic benefit.

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Part V
Cell Death

Chapter 18
Role of Par-4 in Prostate Cancer

Nidhi Shukla, Nikhil K. Hebbar, and Vivek M. Rangnekar

Abstract Prostate apoptosis response-4 (Par-4) is a tumor suppressor protein that induces apoptosis selectively in cancer cells. This specificity toward cancer cells is attributed to the effector domain of Par-4 known as "Selective for apoptosis in cancer cells" (SAC) domain. Par-4 sensitizes cells to the action of diverse apoptotic stimuli that ultimately causes tumor regression. Recent studies have shown that the Par-4 protein is spontaneously secreted by normal and cancer cells, and extracellular Par-4 induces apoptosis by interacting with the cell-surface receptor Glucose-regulated protein 78 (GRP78). GRP78 is a resident protein in the endoplasmic reticulum (ER) that selectively translocates to the surface of cancer cells. This article emphasizes the role of Par-4, as well as the SAC in apoptosis and tumor resistance in mice. SAC transgenic mice, which are resistant to spontaneous as well as autochthonous tumors are described, along with mechanistic insights gained from the interaction of Par-4/SAC and GRP78. The cancer-selective traits of Par-4/SAC make it an ideal choice for cancer therapeutics.

Introduction

Prostate cancer is one of the most commonly occurring forms of cancer, and the second leading cause of cancer related deaths among men in the USA [1]. Prostate cancer begins as a localized lesion and progresses into more advanced and

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metastatic disease. The primary prostate tumor can be subjected to surgical excision and/or treated with androgen deprivation and conventional modes of therapy, such as radiation. However, treatment of advanced disease poses many challenges owing to the loss of androgen dependence, and presence of micro-metastases at the bone at the time of initial presentation. In this chapter, we describe a tumor suppressor protein, Prostate apoptosis response-4 (Par-4), which induces apoptosis in cancer cells without harming the normal cells. Although there are a number of proteins that have been identified as tumor suppressors, the cancer selective feature of Par-4 makes it unique for advanced prostate cancer therapy. An overview of the identification and structure of Par-4, as well as its functional characteristics, is provided below.

Identification of Par-4

Prostate tumors consist of a mixed population of androgen-dependent and androgen-independent cells. Androgen-dependent cells require androgens for their survival and proliferation. In the absence of androgens, these cells undergo apoptosis due to an increase in the level of calcium ions, ultimately leading to tumor regression. This feature is linked to the underlying basis of androgen-ablation therapy, which is the primary treatment method for advanced prostate cancer, where nearly 80 % of patients show an initial response. But this approach selectively targets the androgen-dependent cells, and studies show that more than 50 % of patients can develop androgen-independent disease (termed castration-resistant prostate cancer) within 5 years of treatment [2]. Androgen-independent cells fail to exhibit elevated levels of Ca^{2+} in the absence of hormone, and thus are resistant to apoptosis. However, apoptosis can be induced in these cells by forcing an increase in the levels of Ca^{2+} using ionophores [3]. As an increase in calcium levels is a common principle for apoptosis in both the cell types, we performed a genetic analysis to identify the genes that are activated in response to elevated intracellular Ca^{2+}. The strategy involved treatment of AT-3 androgen-independent rat prostate cancer cells with ionomycin, an ionophore that promotes Ca^{2+} influx into the cells. A set of immediate early genes, designated as Prostate Apoptosis Response (Par), were identified with the help of subtractive hybridization and differential screening studies. Further studies revealed that one of the Par genes was upregulated in response to apoptosis but not by other cellular processes such as necrosis, growth arrest, growth stimulation, or oxidative stress. This gene was named “Prostate Apoptosis Response-4” (Par-4). Nucleotide sequencing studies indicated that Par-4 is not related to the other Par genes. In addition Par-4 expression was also observed upon castration of rats in androgen-dependent cells of the ventral prostate. Interestingly, Par-4 expression was not induced after castration in organs such as kidney and liver which do not undergo apoptosis in response to androgen ablation. Collectively, these observations identified Par-4 as an apoptosis-associated gene [4].

Structure of Par-4

Par-4 was rediscovered through yeast-two hybrid analysis as an interactive partner of Wilm's tumor protein (WT1) [5] and atypical protein kinase C (aPKC) [6]. The human *Par-4* gene consists of seven exons and six introns [7]; it has been mapped to the minus strand of chromosome 12q21 [8]. Human Par-4 protein consists of 342 amino acids (aa), whereas mouse Par-4 has 333aa and rat Par-4 has 332aa, ranging from approximately 38 kDa to 42 kDa in molecular weight. Par-4 is expressed in nearly all the normal tissues of humans, mice, horses, pigs, and cows. Detailed structural analysis led to the identification of two putative nuclear localization sequences (NLS) at the amino-terminal that were termed NLS-1 (aa 20–25) and a bipartite NLS-2 (aa 137–153) and nuclear export sequence (NES), as well as leucine zipper (LZ- aa 290–332) domain at the carboxy-terminus [9]. Further studies showed that the LZ domain was required for the binding of Par-4 with its interactive partners, and NLS-2 was critical for the entry of Par-4 into the nucleus. Analysis of amino-terminal and carboxy-terminal deletion mutants of Par-4 led to the discovery of a unique core domain (aa 137–195) responsible for inducing apoptosis specifically in cancer cells. This domain was named the SAC domain (Selective for Apoptosis in Cancer cells). All these domains have been shown to be 100 % conserved among human, rat, and mouse homologs of Par-4. Apart from these domains, Par-4 protein also contains several potential phosphorylation sites for protein kinases, such as protein kinase C (PKC), protein kinase A (PKA), and Akt [9, 10].

Par-4 is generally present in low amounts in differentiating cells, such as epithelial cells of the mammary gland, neurons, smooth muscle cells, and retinal cells, suggesting that Par-4 is not involved in cell growth and differentiation [11]. The idea of Par-4 being primarily a pro-apoptotic protein is further strengthened by the observation of elevated Par-4 expression in dying cells of degenerating neurons and prostate ductal cells of castrated rats [9]. In normal cells Par-4 generally resides in the cytoplasm, but in cancer cells it can readily translocate to the nucleus and induces apoptosis. Recent studies have discovered that mammalian cells secrete Par-4 protein into the serum, which induces apoptosis in cancer cells via its interaction with surface protein GRP78 [12].

Binding Partners of Par-4

Par-4 interacts with several proteins through its LZ domain at the carboxy-terminus. One such protein is protein kinase C-ζ (ζPKC) that belongs to the atypical protein kinase C (aPKC) family. ζPKC plays an important role in cell growth and cell survival by activating nuclear factor-kappa B (NF-κB) and activator protein-1(AP-1) pathways [13]. Par-4 binds to the zinc finger domain of ζPKC; this interaction of Par-4 and ζPKC was confirmed through yeast-two hybrid studies. Certain stimuli,

such as ionizing radiation or tumor necrosis factor (TNF), promote the binding of Par-4 to ζPKC, and this interaction eventually reduces the enzymatic action of ζPKC due to conformational changes in ζPKCζ, ultimately leading to apoptosis [6, 14]. The reduction in ζPKC activity mechanistically leads to inhibition of IκB kinase (IKK) in the cytoplasm and NF-κB in the nucleus. Recent studies have also discovered a ternary complex that includes an adaptor protein, p62, bound to Par-4/ζPKC. As a result of the interaction of p62 with the Par-4/ζPKC complex, the inhibitory effect of Par-4 on ζPKC is attenuated, and this ultimately promotes cell survival [15].

Another protein that binds to Par-4 is WT-1, a tumor suppressor associated with Wilm's tumor [16]. In nearly 10 % of Wilm's tumor patients, this gene shows homozygous mutation, and thus far this is the only gene known to be involved in the progression of this pediatric tumor. WT-1 induces the transcriptional activity of Bcl-2, a proto-oncogene, first identified in human follicular B cell lymphoma [17] and now known to generally promote cell survival. Over-expression of Bcl-2 inhibits the apoptotic response to chemotherapeutic agents. Since Bcl-2 acts as a pro-survival factor in diverse tumors, Bcl-2 is a target for many anticancer drugs [18]. Par-4 binds to the zinc finger domain of WT-1 and downregulates the Bcl-2 promoter [19], which makes it a promising candidate for targeting cancers associated with over-expression of Bcl-2 [20].

Recent studies have discovered another binding partner of Par-4, a cell survival kinase known as Akt1 or protein kinase B [21]. Akt1 levels are generally high in cancer cells either due to the loss of function of the tumor suppressor gene PTEN or because of constitutive activation of phosphoinositide 3-kinase (PI3K) [22]. Akt1 binds to the LZ of endogenous Par-4 and inactivates it by phosphorylation. The Akt1 phosphorylation site in human Par-4 is at S230, in mouse Par-4 at S231, and in rat Par-4 at S249. The phosphorylation of Par-4 by Akt1 allows a chaperone protein 14-3-3 to bind Par-4 and retain it in the cytoplasm away from its nuclear targets such as NF-κB [23].

Another important binding partner of Par-4 is topoisomerase-1 (TOP1) [24]. Par-4 interacts with TOP1 via the LZ domain and sequesters TOP1, thereby preventing it from binding to the DNA. Consequently, TOP1-regulated DNA relaxation, and associated functions including transcriptional activation of TATA-based promoters, is inactivated. This Par-4 has been shown to inhibit NF-κB activity and induce S-phase growth arrest but not apoptosis [24]. Other binding partners of Par-4 include DAP-like kinase or ZIP kinase (Dlk/ZIP kinase) [25]. The binding of Par-4 and Dlk was identified through a yeast-two hybrid assay and by GST pull-down experiments. There is some evidence suggesting that Dlk is inactive in the nucleus, and after binding to the Par-4, it translocates to the cytoplasm where it is active [26, 27].

Par-4 is a Tumor Suppressor Protein

For a gene to be classified as a tumor suppressor, it should satisfy two main characteristics, which include (1) loss of function of the gene should result in a cancer phenotype, and (2) inactivation of the gene in vivo should enhance the tumor

initiation, growth, or progression [28]. Par-4 is downregulated in a variety of cancers, such as renal cell carcinoma [29], neuroblastoma [30], leukemia, and chronic lymphocytic leukemia [31], and is inactivated in human endometrial cancer [32]. Par-4 is located at chromosome 12q21, which is generally unstable in gastric and pancreatic cancer [33, 34]. This region also contributes to the development of Wilm's tumor [8]. Par-4 is not downregulated or mutated in prostate cancer but is held inactive in the cytoplasm by Akt1 and 14-3-3, which prevents its nuclear translocation, thereby inhibiting it's apoptotic action [23].

Par-4 knockout mice develop spontaneous tumors in many organs, such as liver, lung, and endometrium, and also the precursor lesion, prostatic intraepithelial neoplasia (PIN). Heterozygous as well as homozygous loss of Par-4 produces the same frequency of tumors and PIN. Par-4 knockout mice have a shorter life span compared to wild type as they die because of spontaneous tumors [35].

One of the most commonly mutated genes in human cancers is the *Ras* oncogene [36]. Mutations in *Ras* lead to active signaling that specifically upregulates pro-survival factors such as NF-κB. The oncogenic effect of *Ras* results from its downregulation of the components of antiapoptotic pathways [37–39]. Pancreatic cancer specimens with *K-Ras* mutations exhibit Par-4 downregulation [40, 41]. Par-4 is downregulated by *Ras* in a variety of cell types through the MEK-ERK pathway, and this action of Ras is a crucial step toward Ras-induced transformation. Restoration of Par-4 levels either by MEK inhibition or by stable expression of ectopic Par-4 abrogates cellular transformation and induces apoptosis [20, 42, 43].

Mechanisms of Par-4-Induced Apoptosis

Par-4 executes its pro-apoptotic or anticancer function by two co-parallel mechanisms: (1) activation of components of the cell-death system and (2) inhibition of pro-survival factors. There are two major signaling pathways for programmed cell death: the extrinsic pathway and the intrinsic pathway. An extrinsic pathway starts through ligation of specific ligands to the cell death receptors, thereby inducing apoptosis through activation of death domain proteins and the caspase-8/caspase-3 cascade. Par-4 activates the extrinsic pathway by translocating Fas and Fas ligand to the plasma membrane, which leads to the formation of a complex known as death inducing signaling complex (DISC). DISC is mainly involved in the induction of apoptosis and is made up of Fas, the death domain protein known as Fas-associated death domain (FADD) and pro-caspase 8. Translocation of Fas and Fas ligand to the plasma membrane by Par-4 is limited in hormone-independent cancer cells where Par-4 over-expression is sufficient to induce apoptosis; by contrast this effect is not present in the hormone-dependent cancer cells [44]. The intrinsic pathway is initiated by cellular or environmental stress signals that alter mitochondrial function. Signals that induce mitochondrial dysfunction include ionizing and UV radiation or substances that are responsible for increasing the intracellular Ca^{2+} concentration [45].

In normal tissues Par-4 is primarily located in the cytoplasm, but in cancer cells it readily translocates to the nucleus. Deletion analysis has demonstrated that the nuclear localization sequence 2 (NLS2) domain and not the NLS1 at amino-terminal of Par-4 is essential for nuclear translocation, which is a crucial step in the process of apoptosis by Par-4. There are two necessary steps for Par-4-induced apoptosis: its nuclear entry and phosphorylation at T155 residue by PKA. Normal cells generally contain low levels of PKA activity, and Par-4 does not get phosphorylated and does not induce apoptosis. Cancer cells generally have relatively higher PKA activity, and hence Par-4 gets phosphorylated and readily translocates to the nucleus to induce apoptosis. Phosphorylation by PKA is a crucial step toward apoptosis by Par-4 since mutation at T155 completely abrogates nuclear entry and the apoptotic function of Par-4 [46]. Cancer cells that are resistant to apoptosis by Par-4 exhibit Par-4 phosphorylation by PKA, but Par-4 is held back in the cytoplasm by pro-survival factors such as Akt1 [23]. These observations indicate the importance of nuclear entry for Par-4 to exert its apoptotic function [47].

Par-4 also exerts its apoptotic effects by inhibiting pro-survival factors. NF-κB is one such factor that plays an important role in oncogenesis [48]. NF-κB confers resistance to cell death by activating pro-survival genes including Bcl2 family members such as Bcl2, NR13, Bcl-xL, and antiapoptotic proteins such as cellular inhibitor of apoptosis 1 and 2 (cIAP1 and 2) that acts as a caspase inhibitor and X-linked inhibitor of apoptosis (XIAP) that acts as a cell protector from TNF-induced apoptosis [49]. Par-4 inhibits NF-κB activation by inhibiting its nuclear translocation, as well as by inhibiting its transcriptional activity in the nucleus [10, 14, 24].

Identification of the Effector Domain of Par-4

As indicated above the SAC domain of Par-4 is selective in inducing apoptosis in diverse cancer cells but not in normal cells. SAC exerts its apoptotic function by a mechanism similar to that of Par-4, including inhibition of pro-survival NF-κB activity. The SAC domain contains NLS2 to facilitate nuclear entry and the T155 phosphorylation site for Par-4 activation. The SAC sequence is 100 % conserved among humans, rats, and mice [10]. The SAC domain lacks the LZ domain that is important for interaction with other proteins, hence it does not bind to Akt1 and is not prone to Akt1-mediated phosphorylation and inactivation. Therefore, in comparison to Par-4, SAC induces apoptosis in a wider range of cancer cells including those with high levels of Akt1 activity. However, in normal or immortalized cells, SAC localizes to the nucleus but is not able to induce apoptosis, since it needs phosphorylation at T155 residue by PKA activity, which is generally low in these cells [47]. However, apoptosis by SAC in normal cells can be induced by artificially elevating PKA activity either by cAMP or by over-expression of catalytic subunit of PKA. As most anticancer drugs primarily induce apoptosis in cancer cells and also harm normal cells, development of a tumor-specific drug is a major

area for cancer therapy investigations. Since SAC selectively induces apoptosis only in cancer cells but not in normal cells; it is a promising candidate for anticancer therapy.

SAC Transgenic Mice

Mice are widely used as a model for studies related to cancer progression, because the human and mouse genomes show significant homology and have similar tumor-related pathways. Moreover, like humans, mice develop tumors especially as they age. Transgenic mice that ubiquitously express the SAC domain were generated to analyze its functional efficacy. As the SAC domain is quite small, we tagged it with enhanced green fluorescent protein (eGFP) to generate a transgenic mouse. A plasmid vector pCAGGS was chosen because of its ability to constitutively express a transgene [50]. It has a cytomegalovirus (CMV) enhancer and a chicken β-actin promoter located upstream of the multiple cloning site (MCS) region, allowing ubiquitous expression of the transgene. It also contains a rabbit β-globin polyadenylic acid sequence located downstream from the MCS region. SAC transgenic mice were generated by cloning a DNA fragment, containing the SAC domain and tagged with eGFP at the carboxy-terminal, into the pCAGGS vector. Similarly, eGFP transgenic control mice were generated by cloning the eGFP coding sequence into the pCAGGS vector. These mice were produced using the B6C3F1 background. Ubiquitous expression of SAC-GFP and GFP proteins was confirmed by Western blot analysis of different tissues with the GFP antibody [51].

SAC Transgenic Mice Show Increased Resistance to Spontaneous Tumors

It had been reported previously that B6C3F1 mice develop hepatocarcinomas and lymphomas with increasing age [52]. The GFP transgenic mice as well as littermate control mice developed a high frequency of lung and splenic tumors at the age of 18 months, whereas SAC transgenic mice were completely devoid of tumors. Histopathological examination of liver sections from both control and GFP mice showed loss of normal liver structure, presence of necrotic areas, enlarged hepatocytes with irregular nuclei, and vascular invasion in some tumors, whereas sections from the SAC transgenic mice showed normal liver architecture. Similarly, the spleens of GRP transgenic and littermate control mice were enlarged, and the basic architecture was destroyed. The lymphocytes had condensed chromatin and irregular nuclear spots, whereas SAC transgenic mice showed regular splenic architecture. These results indicate that the SAC transgene suppresses the growth and development of spontaneous tumors in the liver and spleen of SAC transgenic mice.

These characteristics of tumor resistance were also observed through many generations indicating successful and stable inheritance of SAC [51].

SAC Transgenic Mice Show Normal Development

Certain tumor suppressor genes target other vital processes including aging and senescence along with their primary functions [53]. For example, a transgenic mouse in which the amino-terminal of p53 is truncated shows increased tumor resistance but also shows premature aging [54, 55]. Therefore, SAC transgenic mice were monitored for any abnormalities in characteristics such as body weight, age, fertility, and life span. The SAC transgenic mice were of the same weight as the GFP control mice, as well as non-transgenic littermate controls. They were developmentally normal, and their birth ratios were similar to the controls. Interestingly, SAC transgenic mice lived longer than the control mice, clearly indicating that the SAC transgene does not affect growth, age, or fertility of the animals [51]. This evidence supports the concept that resistance toward cancer can be increased without altering other vital functions [51].

SAC Transgenic Mice Show Increased Resistance to Prostate Tumor Growth

In order to investigate whether the SAC domain can inhibit the growth of oncogene-induced tumors, SAC or GFP transgenic mice were crossed with transgenic adenocarcinoma of the mouse prostate (TRAMP) mice [56]. TRAMP mice spontaneously produce adenocarcinomas of the mouse prostate. Crosses were made to generate mice with different genetic backgrounds such as $SAC^{-/-}/TRAMP^{+/-}$, $SAC^{+/-}/TRAMP^{+/-}$, $SAC^{+/-}/TRAMP^{-/-}$, and $GFP^{+/-}/TRAMP^{+/-}$ and were closely observed for prostate tumor progression. Interestingly, by the age of 3 months nearly 60–80 % of control $GFP^{+/-}/TRAMP^{+/-}$ and $SAC^{-/-}/TRAMP^{+/-}$ mice developed high grade PIN, around 14–16 % of animals developed adenocarcinoma of the prostate and by the age of 6 months, eventually 100 % of these mice developed adenocarcinoma of the prostate. In contrast, at the age of 3 months only 50 % of $SAC^{+/-}/TRAMP^{+/-}$ mice developed high-grade PIN, but none of them developed adenocarcinoma. By the age of 6 months, only 21.4 % of the mice developed adenocarcinoma, whereas 28.6 % mice did not develop tumors. These observations indicate that the SAC domain transgene inhibits TRAMP tumor progression. Further, to look for expression of the SAC domain in these tumors, immunohistochemical analysis on prostate sections was carried out. GFP was expressed in the PIN lesions, as well as in the adenocarcinoma of the prostate of $GFP^{+/-}/TRAMP^{+/-}$ mice at 3 and 6 months of age, whereas $SAC^{+/-}/TRAMP^{+/-}$ mice expressed the SAC transgene in normal cells but not within

the PIN lesion or in the adenocarcinoma. The $SAC^{+/-}/TRAMP^{-/-}$ control mice continued to show SAC expression in the prostate at 3 and 6 months of age. Also, 80 % of the $SAC^{+/-}/TRAMP^{+/-}$ mice developed adenocarcinoma of the prostate, and 20 % developed PIN lesions at the age of 12 months. Immunohistochemical analysis showed a loss of the SAC domain from all of these tumors. Together these data suggest that the SAC transgene must be downregulated before development of adenocarcinoma of the prostate and that for the progression of disease it should lose its tumor suppressor function [51].

Apoptosis by Secreted Par-4

Recently, a new mechanism of Par-4 activity has been discovered. Par-4, which is secreted by normal, immortalized, as well as cancer cells, selectively induces apoptosis in cancer cells through its cell surface receptor, glucose-regulated protein of 78 Kd (GRP78) [12, 57].

Protein trafficking from the endoplasmic reticulum (ER) to cis-Golgi cisternae [58] results in the accumulation of secretory proteins within the ER and is a crucial step in the secretion of proteins by the classical pathway. An antiviral antibiotic brefeldin A (BFA) can block this pathway. Treatment of cells with BFA results in inhibition of Par-4 secretion, suggesting that secretion of Par-4 takes place through a BFA-sensitive classical pathway involving the ER–Golgi network [12].

The ER is an essential organelle for cell viability and a major intracellular store for calcium and the production site for lipids and sterols. It also functions as a cellular organelle for the synthesis, assembly, and glycosylation of proteins that are designated for secretion or for transport to the cell surface. ER homeostasis is disturbed by the inhibition of N-linked glycosylation, depletion of calcium in ER lumen, over-expression of some wild-type proteins, expression of mutant proteins/protein subunits, or reduction of disulfide bonds that results in stress signaling. These disturbances lead to the activation of unfolded proteins and subsequent induction of an unfolded protein response (UPR), a mechanism conserved from yeast to humans [59].

The transcription factor CHOP/GADD153 (GEBP homologous protein/growth arrest and DNA damage inducible) is induced at the transcriptional level in response to ER stress. CHOP may negatively regulate cell growth and induce apoptosis [60], and over-expression of CHOP can lead to cell cycle arrest. Other inducers of ER stress include tunicamycin (TU), an inhibitor of N-linked glycosylation [61], and thapsigargin (TG), an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{+2} (SERCA) ATPase [62]. Treatment of cells with ER stress inducers TU and TG enhance the secretion of Par- 4, and also cause upregulation of GRP78 and CHOP/GADD153. Upon further characterization of the mechanism of apoptosis induced by secreted Par-4, an integral role of the ER chaperone protein GRP78 in extracellular Par-4 and SAC-mediated apoptosis was discovered [12].

GRP78, also termed as BiP (immunoglobin heavy chain binding protein), was discovered as a protein that is induced in response to glucose starvation [63]. GRP78 belongs to the protein family HSP70, which plays an important role in oncogenesis. Apart from functioning as a key element in proper protein folding, targeting misfolded proteins, and promoting cell growth, it also functions as an ER stress signaling regulator by binding with Ca^{2+} and as antiapoptotic protein [64]. It is generally present in low amounts in organs such as lung, heart, and brain but is elevated in various tumors especially those with aggressive characteristics. GRP78 is present on the surface of cancer cells [65] and acts as a pro-survival factor by binding to the extracellular signaling protein Cripto [66] and proteinase inhibitor alpha 2-macroglobulin [67]. However, contradictory to this function, it also acts as a pro-apoptotic factor by binding to the Kringle 5 [68], an angiogenesis inhibitor and Par-4, a tumor-suppressor protein [12].

The interaction between GRP78 and Par-4 has been confirmed by co-immunoprecipitation studies that showed colocalization of GRP78 and Par-4 at the plasma membrane. Knocking down of GRP78 expression with RNAi or neutralization of Par-4 in the conditioned medium with recombinant GRP78 showed inhibition of apoptosis by extracellular Par-4. Thus, GRP78 acts as a cell surface receptor for extracellular (secreted) Par-4, and the interaction between Par-4 and GRP78 is essential for the apoptotic signaling [12].

Extracellular Par-4 acting via the GRP78 receptor induces apoptosis through a FADD-dependent pathway. The adapter protein FADD then recruits the apoptotic machinery by inducing the caspase pathways. ER stress also plays an important role in this process by transclocating GRP78 to the cell surface and also by activating the extracellular Par-4 or TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. An ER- resident protein, PKR-like ER Kinase (PERK) is known to mediate apoptosis induced by FADD and caspase-8 pathways [69]. Also, knockdown of PERK by RNA interference (RNAi) results in the inhibition of apoptosis, suggesting its role in the process of apoptosis. This concept was further strengthened by the observation that cells treated either with TRAIL or recombinant Par-4 exhibit higher levels of phospho-PERK. Most interestingly, unlike the previously described binding partners of Par-4, whose interaction with Par-4 is mediated through the carboxy-terminal LZ domain, the interaction between Par-4 and GRP78 is mediated uniquely by the SAC domain [12].

Role of Intracellular Par-4 in Apoptosis by Extracellular Par-4

The correlation between intracellular Par-4, GRP78 and extracellular Par-4 in activating apoptosis has been further confirmed by studies using RNAi [12]. Knocking down Par-4 resulted in decreased translocation of GRP78 to the cell surface, indicating that endogenous Par-4 is required for cell surface expression of GRP78. Par-4 knockdown also resulted in resistance to recombinant Par-4 or SAC-induced apoptosis, but when these knockdown cells were transfected with membrane-directed full length GRP78 (mGRP78), sensitivity to recombinant Par-4 was restored.

By contrast, the N-terminal mutant of GRP78 (mDN), from which 66 amino acids have been deleted from the N terminus, did not support apoptosis mediated by recombinant Par-4. Interestingly, immortalized prostate BPH-1 cells, which are usually resistant to recombinant Par-4, became sensitive to recombinant Par-4-induced apoptosis when transfected with full length GRP78 but not the N-terminal mutant of GRP78 [12]. Collectively, these findings suggest that both intracellular Par-4 and GRP78 play important roles in extracellular Par-4-mediated apoptosis.

Future Directions

Although Par-4 has been studied extensively, there are many questions that still remain unanswered. For instance, the interplay between the androgen receptor (AR) and the regulation of Par-4 has not been adequately addressed. It has been observed that a decrease in AR levels following castration results in a significant increase in Par-4 levels in the prostate. This increase in Par-4 results in Fas/FasL-induced apoptosis [70], suggesting that it could have important implications in patients with prostate cancer. Studies on the regulation of Par-4 by AR may shed more light on the clinical significance of these findings.

Anti-androgenic drugs such as bicalutamide have been shown to be effective in the treatment of prostate cancer by downmodulating the AR pathway [71]. It would be interesting to test whether inhibition of the AR signaling pathway may enhance Par-4-mediated tumor cell apoptosis. The secretion of Par-4 into circulation by AR pathway inhibition is another area that can be exploited to improve clinical outcomes. Moreover, discovering novel, as well as FDA approved agents, that can stimulate the production and enhance the secretion of Par-4 from the normal stroma of the prostate would open new avenues in the treatment of both androgen-dependent and -independent prostate cancer. The use of such compounds may allow targeting of the primary tumor, as well as the metastatic cells in circulation, thereby preventing metastases. The effect of these compounds needs to be carefully delineated, with or without androgen deprivation therapy. Such studies may uncover the underlying mechanisms of Par-4 action and also improve our ability to treat cancer patients with minimal toxicity and distress.

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Chapter 19
Autophagy and Prostate Cancer Therapeutics

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Abstract Autophagy or self-eating is an evolutionarily conserved process whereby cells, in response to stress conditions, use lysosomal-mediated degradation of long-lived proteins and retired organelles to regenerate energy. It protects cells from harsh conditions and prolongs cell survival. Cancer therapeutics induce a variety of stresses in tumor cells including nutritional starvation, DNA damage, ER stress, and ROS generation. Not surprisingly, the great majority of cancer therapeutics also induce autophagy. As such, autophagy becomes an inseparable part of cancer therapy and its modulation, and thus deserve attention. In this review, we discuss the current prostate cancer therapies, the cell biology and detection method of autophagy, the relationship of autophagy to apoptosis and necroptosis, and autophagy modulation in experimental prostate cancer therapies. Finally, we provide a

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comprehensive summary of the autophagy characteristics (induction and function) of experimental and clinically tested prostate cancer treatments, as well as current clinical trials involving autophagy modulators.

Abbreviations

2-DG	2-deoxyglucose
4EBP	EIF4EBP, eukaryotic translation initiation factor 4E binding protein 1
4-IBP	*N*-(*N*-benzylpiperidin-4-YL)-4-iodobenzamide
ADI	Arginine deiminase
AFFIRM	Atrial Fibrillation Follow-up Investigation of Rhythm Management
AIF	Apoptosis inducing factor
AMPK	AMP-activated protein kinase
AR	Androgen receptor
ASS	Argininosuccinate synthetase
ATG	Autophagy-related gene
ATO	Arsenic trioxide
BH3	Bcl-2 homology domain 3
CAD	Caspase-activated DNase
CQ	Chloroquine
CRPC	Castrate-resistant prostate cancer
CYP17	Cytochrome P450 17A1
DMOG	Dimethyloxaloylglycine
DNA	Deoxyribonucleic acid
DP	2,2′-Dipyridyl
DRAM	Damage-regulated-autophagy-modulator
DRP1	Dynamin related protein 1
ENDO-G	Endonuclease G
ER	Endoplasmic reticulum
FH	Fumarate hydratase
GFP	Green fluorescent protein
GnRH	Gonadotropin-release hormone
HCQ	Hydroxychloroquine
HDAC	Histone deacetylase
HMGB1	High-mobility group protein B1
IAP	Inhibitor of apoptosis
ICMT	Isoprenlycysteine carboxyl methyltransferase
IDH	Isocitrate dehydrogenase
IL6	Interleukin 6
ILK	Integrin-linked kinase
IR	Ionizing radiation
LC3	Light-chain 3
LHRH	Luteinizing hormone-releasing hormone

LPA	Lysophosphatidic acid
MAP	Microtubule-associated protein
mTOR	Mammalian target of rapamycin
PD	Pro-death
PEITC	Phenethyl isothiocyanate
PGAM5	Phosphoglycerate mutase 5
PI3K	Phosphoinositide 3 kinase
PS	Pro-survival
RANK	Receptor activator of nuclear factor kappa-B
RFP	Red fluorescent protein
RIP1	Receptor-interacting protein 1
ROS	Reactive oxygen species
S1P	Sphingosine 1-phosphate
SDH	Succinate dehydrogenase
SFN	Sulforaphane
Src	Sarcoma (a proto-oncogene tyrosine-protein kinase)
TKI	Tyrosine kinase inhibitor
TNFa	Tumor necrosis factor alpha
TSC2	Tuberous sclerosis protein 2

Prostate Cancer Therapeutics

The standard treatment for advanced prostate cancer is androgen deprivation therapy using luteinizing hormone-releasing hormone (LHRH) agonists or antagonists, which initially controls the disease effectively by reducing 95 % of the circulating testosterone (chemical castration). However, after a durable response of 18–24 months, progression to castrate-resistant prostate cancer (CRPC) is almost universal despite achieving castration levels of testosterone. Fewer than 20 % of patients survive beyond 3 years after progression to CRPC. Prior to 2004, standard treatment for CRPC was Mitoxantrone plus prednisone, which reduces symptoms and improves the quality of life but does not prolong life for these patients.

In 2004, the combination regimen of docetaxel/prednisone demonstrated a 2.4-month survival advantage over placebo in two randomized controlled trials and was approved for the management of patients with metastatic CRPC [1, 2]. From 2010 to 2012, advances in the basic sciences led to the development of four new therapies for patients that progressed in the post-docetaxel setting including: (1) sipuleucel-T, an immunotherapeutic, which showed a 4.1-month improvement in median survival; (2) cabazitaxel, a novel taxane that induced G2/M phase cell cycle arrest by stabilizing microtubules, which offered a 2.4-month survival advantage; (3) abiraterone acetate, a CYP17 complex/androgen synthesis inhibitor, which demonstrated a 4-month improvement in overall survival; and (4) denosumab, a monoclonal antibody against RANK ligand, and zoledronate, a bisphosphonate, which were approved for patients with bone metastasis to decrease skeletal-related events.

Most recently, the novel androgen signaling inhibitor enzalutamide (MDV3100) showed a 4.8-month survival benefit over placebo in the AFFIRM (A Study Evaluating the Efficacy and Safety of the Investigational Drug MDV3100) Phase III trial [3]. Enzalutamide is a small molecule with multiple effects on androgen signaling including blockage of testosterone binding to androgen receptor (AR), preventing AR nuclear translocation and DNA binding, and interfering with co-activator recruitment. Given that AR signaling continues to drive cancer progression in the castrate-resistant setting, there is still emphasis in developing novel agents to block extra-gonadal androgen synthesis and AR signaling. TAK-700 and TOK-001 are new CYP17 inhibitors in Phase II clinical trials. A new AR signaling inhibitor ARN-509, with similar properties to enzalutamide, is currently in a Phase I/II trial.

In summary, the current prostate cancer therapy repertoire consists of hormone therapy targeting androgen/AR axis (e.g., LHRH, abiraterone, enzalutamide, ARN-509, TAK-700, and TOK-001), chemotherapy targeting mitosis (e.g., docetaxel, cabazitaxel), and immunotherapy (e.g., sipuleucel-T, denosumab). As discussed below, these therapies utilize distinct death pathways to induce cell killing, which may be exploited to enhance the therapeutic efficacies.

Mechanisms of Cell Death Associated with Therapeutics

Conventional chemotherapeutics and radiation therapy, exploiting the requirement of DNA replication and cell division for tumor growth, rely on damage of DNA or mitotic machinery. These processes trigger mitotic catastrophe, resulting in the death of tumor cells. Those tumor cells that escape death due to checkpoint defectiveness are associated with chromosomal instability and aneuplody [4]. There are multiple ways whereby failure to go through mitosis leads to cell death that are typified by caspase-dependent and independent apoptosis. Apoptosis (program cell death type I) is a process triggered intracellularly by DNA fragmentation and nuclear collapse, eventually leading to the formation of apoptotic bodies where fragmented DNA is segregated by cellular membranes. The key enzymes involved are a family of caspases, which activate caspase-activated DNase (CAD) to fragment DNA [5].

While caspases are key mediators of apoptosis, caspase-independent apoptosis has also been reported. The principal players are mitochondria proteins apoptosis inducing factor (AIF) and Endo-G nuclease, which upon activation, are translocated into the nucleus and execute DNA degradation. Therapeutic resistance often results from mutations in the p53 or other apoptosis mediators. To overcome such resistance, other programmed death pathways and strategies to target them are being sought.

As traditional chemotherapy interferes with cell division non-discriminatorily, it also affects normal replicating cells and thus may introduce serious side effects. An emerging therapeutic strategy is to take advantage of the different metabolic requirements of tumor vs. normal cells and selectively starve the cancer cell to

death. One prominent example is the preference of tumor cells toward glucose and the aerobic glycolysis pathway (e.g., Warburg effect) [6, 7]. Recently, the differential utilization of glutamine, serine, and glycine pathways by tumor cells was also recognized [6]. Specific metabolic deficiencies of tumor cells due to genetic mutations (e.g., isocitrate dehydrogenase (IDH) [8], fumarate hydratase (FH) [9], and succinate dehydrogenase (SDH)) [10] or epigenetic silencing (e.g., argininosuccinate synthetase (ASS)) of rate-limiting components of the metabolic pathway have been identified [11]. For prostate cancer, transcriptional suppression of the arginine synthesis enzyme, ASS, was found in 100 % of tumor samples, rendering them "addicted" to external arginine. These findings have fueled recent interest in developing therapeutic starvation strategies for prostate and other types of cancers. Some of the examples include the use of 2-DG (2-deoxyglucose) to inhibit the glycolysis pathway [12] and the application of arginine deiminase (ADI) for arginine depletion [13]. Androgen deprivation and anti-androgen can also be considered among the starvation therapies. These therapies are often characterized by the appearance of vacuoles, a process called autophagy. Autophagy or self-eating is an evolutionarily conserved process, whereby cells, upon nutritional stress, degrade retired proteins and organelles to regenerate energy and amino acids. The process starts with the formation of double-membrane vesicles, which engulf the wasted proteins and organelles, and fusion with lysosomes for degradation. Although autophagy allows cells to survive under stress conditions, excessive or prolonged autophagy has been postulated to be a mechanism of cell death, i.e., programmed cell death type II. The signals that shift autophagy from cell survival to the cell death pathway remain unknown. Although the details of autophagic cell death remain largely unclear, several hypotheses including lysosomal rupture and cathepsin release have been proposed. It is, however, generally viewed that autophagic death is intrinsically distinct from apoptosis, and thus can be used as an alternative pathway to overcome the apoptosis resistance of tumor cells. As will be described below, most experimental anti-prostate cancer agents induce autophagy as a cellular stress response and, depending on the treatment regime, autophagy functions either as a pro-survival (PS) or pro-death (PD) factor.

A third cell death mechanism, necrosis or necroptosis (programmed necrosis), is characterized by the loss of plasma membrane potential due to energy depletion and, like classical apoptosis, mitochondria dysfunction [14]. Indeed, the caspase-independent apoptosis mediated by AIF/ENDO-G release is sometimes considered as necroptosis [15]. Recently, mitochondria fission mediated by RIP1 and 3 (receptor interacting protein 1 and 3), PGAM5, a mitochondria-associated protein phosphatase, and DRP1, dynamin related protein 1, has been found to be one pathway involved in necroptosis induced by TNFα [16]. The process involves the assembly of the RIP/PGEM5 complex at the mitochondrial membrane, which then dephosphorylates and recruits Drp1 to mitochondria, leading to mitochondria fragmentation, with eventual plasma and nuclear membrane breakdown. The downstream effectors for necroptosis are unknown, but likely involve ROS and calcium induction. The identification of necroptosis generally relies on the measurement of the

permeability of permeability of propidium iodide and the release of nuclear protein HMGB1. Very little is known about necrosis induced by prostate cancer therapeutics.

While apoptosis, autophagic death, and necrosis are three distinct cellular death mechanisms, they are interconnected. For instance, p53, depending on its cellular location, modulates all three pathways [17, 18]. Bcl-2 is a key modulator of mitochondrial pore formation involved in apoptosis, yet also interacts with Beclin 1, a critical component for autophagy, thereby regulating both apoptosis and autophagy pathways [19]. Bax and BH3-only molecules are implicated in mitochondrial membrane permeablization for both apoptosis and necrosis. Calpain, activated by autophagy, cleaves Atg5, a component involved in autophagosome formation, into a form which goes to mitochondria and induces apoptosis [20–22]. This is one example of how starting with autophagy can end up with apoptosis. On the other hand, inhibiting autophagy has been shown to induce caspase-dependent apoptosis in certain contexts [23], and vice versa [24]. Lian et al. [25] reported that the treatment of prostate cancer cells with (−)- gossypol and Sorafenib resulted in death by apoptosis (in DU145 cells) or autophagy (in PC3 cells) in a cell context-dependent manner related to the amount of Bax expression in the cell. Inhibition of the non-dominant death pathway (e.g., autophagy in DU145) enhances the efficacy to killing mediated by others (e.g., apoptosis in DU145), clearly illustrating the antagonism between these two pathways. Thus, in considering cell death induced by therapeutics, all three mechanisms need to be investigated. In the present review, we will focus on autophagy.

Autophagy Detection and Measurement

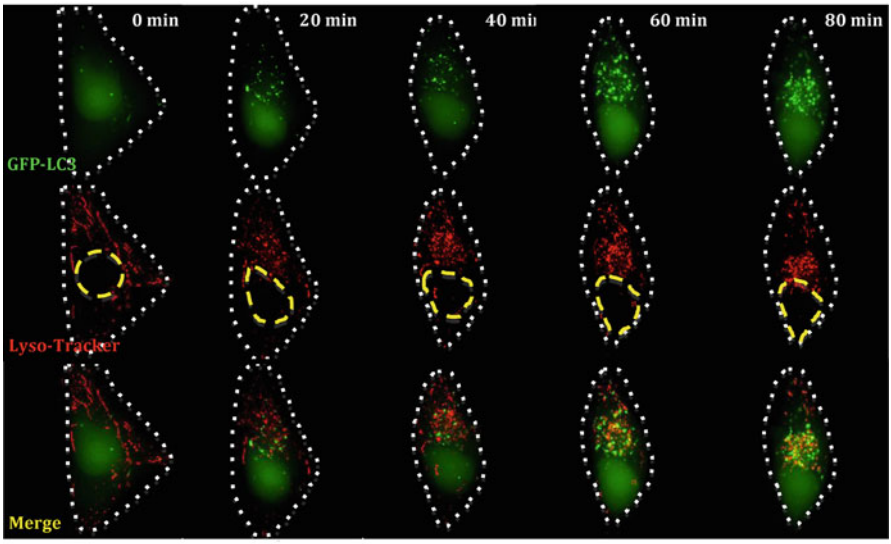
Although autophagy was first characterized in yeast nearly 50 years ago, the realization that autophagy could have an important role in different disease processes has only recently sparked much interest within the biomedical research community. However, of the many tools and techniques that were established to monitor autophagy in yeast, relatively few can be applied to mammalian systems. The most prominent biomarker associated with autophagy in eukaryotic cells is microtubule-associated protein (MAP) 1A/1B light chain 3A or, simply, LC3. LC3 is initially synthesized in the unprocessed form, proLC3, which is proteolytically cleaved into LC3-I. LC3-I is localized in the cytoplasm, but upon induction of autophagy, it is lipidated into LC3-II and inserted into the autophagosome membrane (presumably at the site of autophagosome nucleation) [26]. The conversion of LC3-I to LC3-II can be monitored by Western blot analysis as a measure of autophagosome formation. As autophagy proceeds, the absolute intensity of both LC3-I and II decreases, due to fusion of the autophagosome and lysosome, after which degradation occurs [27]. In addition to LC3, the degradation of P62/SQSTEM1, a linker protein that connects LC3 and ubiquitinated substrates, is also commonly used as a marker to monitor autophagy flux [28, 29]. Alternatively, in cells that contain LC3 that is fluorescently labeled, either by direct antibody staining or by incorporation and expression of

green fluorescent protein (GFP)-coupled LC3, the induction of autophagy can readily be detected and visualized by the prominent change from diffuse cytoplasmic to bright, punctate fluorescence in the cytoplasm. This technique can be applied to nearly all cell types, either live or fixed, and can even be combined with flow cytometry to monitor the induction of autophagy in live cells. However, this approach is only semiquantitative as conventional fluorescence microscopy lacks the resolution to image structures (such as nascent autophagosomes) that are smaller than 0.2 ~ 0.5 μm; and electron microscopy is not practical for imaging dynamic biological processes. In recent years, a new generation of optical imaging technologies has emerged—all capable of fluorescence imaging at higher resolutions. Some of these technologies allow the detection of 100× more autophagosomes, providing a more accurate measurement of autophagosome growth.

High-resolution microscopic imaging is essential for determining when, where, and how the key steps of autophagy occur, and with strategic labeling of other molecular components (such as labeling autophagosome with LC3-GFP and lysosome with Lamp1-RFP or LysoTracker Red (Invitrogen)), it is now possible to visualize and quantify the entire process of autophagy over time with unprecedented detail. For example, using a 3-dimensional imaging platform based on widefield fluorescence deconvolution microscopy (DeltaVision by Applied Precision, a GE Healthcare Company) that utilizes a portable microfluidic perfusion viewing chamber (ONIX by CellASIC), one is able to fully monitor the biophysical changes that occur in live prostate cancer cells CWR22R*V*1 early upon autophagy induction by rapamycin (Fig. 19.1). A rapid formation and proliferation of autophagosomes (often to the point of displacing cell nuclei away from the center) has been consistently observed. Over time, these autophagosomes appear to fuse and grow larger—eventually combining with lysosomes to form autolysosome (Fig. 19.1. objects indicated in yellow) such that the contents of the autophagosomes will be degraded. Both nonimage-based and image-based techniques have provided tremendous insights into the process and function of autophagy. We expect that new emerging technology and improvements on current technology will enable us to address critical questions such as the principal scale at which autophagy typically occurs in normal physiology; the intracellular source(s) of material for the synthesis of autophagosomes; the kinetics of autophagy, and its relation to cell death.

Autophagy and Prostate Cancer Therapeutics

As shown in Table 19.1, autophagy, a cellular stress response, is induced by a variety of cancer therapies including chemotherapy, radiotherapy, targeted therapy, and starvation therapy. For prostate cancer, the foremost therapeutic intervention is androgen ablation or anti-androgen treatment. It is known that androgen can suppress serum-starvation-induced autophagy [50, 98], and that anti-androgen treatment or androgen withdrawal in the presence of hypoxia leads to autophagy induction [99] (unpublished data). This is understandable, as depleting androgen is



Time (min)	0	20	40	60	80
Autophagosome Count	49	125	152	167	203

Fig. 19.1 Image sequence showing prostate cancer cell CWR22R*V*1 cells treated with rapamycin from 0 to 80 min. The white dotted line represents the outline of the cell, and the yellow dotted line represents the position of the nucleus. The number of autophagysome (green) and lysosome (red) increases over time, and the number of colocalization (yellow) also increases over time. Autophagosome counts were extracted by analyzing the image sequence as a demonstration that the image data is quantifiable.

a form of nutritional starvation and stress induction. The LNCaP cell line, a prototype of androgen-responsive model, undergoes neuroendocrine differentiation upon androgen withdrawal or IL-6 treatment [100], and, autophagy is responsible for IL-6-induced neuroendocrine differentiation [101]. In addition to hormone therapy, a variety of anti-prostate cancer treatments have been shown to induce or repress autophagy (+ or – in Table 19.1). As shown, most of these agents induce autophagy; notable exceptions include metformin, docetaxel [56], nelfinavir [45, 102], 4-IBP [83], and LPA [103] which suppress autophagy. Those which induce autophagy do so by a variety of mechanisms including metabolic stress, induction of ER stress, generation of ROS, and DNA damage (Fig. 19.2). These mechanisms are not mutually exclusive and are interconnected. A word of caution in reading the literature is the tendency to overestimate autophagy induction, by virtue of the appearance of autophagosomes or the conversion into LC3-II. Reagents that block autophagy such as chloroquine and bafilomycin could be mistakenly scored as an autophagy inducer, if autophagosome formation is the sole criterion. For those studies in which mechanisms were investigated, the activation of AMPK (AMP-dependent protein kinase) and p53 as well as the suppression of PI3K/AKT/mTOR are the predominant modes of autophagy induction. AMPK activation leads to phosphorylation of TSC2 and

Table 19.1 Review of published literatures on autophagy and prostate

Treatments	Cell lines	Autophagy	Apoptosis	Citations
Small molecules				
Oridonin (ORI)	PC-3 and LNCaP	+, PS*	+	[30]
Oridonin	PC-3	+, PS		[31]
Monascuspiloin	LNCaP and PC-3	+, PD* for PC3	+ for LNCaP	[32]
Alternol	C4-2B and RWPE-1	+, PD	+	[33]
Geraniol	PC-3	+, PD*	+	[34]
Celastrol	PC-3	+, PD*	+	[35]
Curcumin	CWR22rv1	+, PD	+ (Caspase dependent)	[36]
Penta-galloyl-glucose (PGG)	DU145, PC3, and LNCaP	+, PS*	–	[37]
Licorice or licochalcone-A	LNCaP	+, ND	+	[38]
Prenylflavonoids (X, IX, 8PN)	PC-3 and DU145	+, PD	–	[39]
Statin and/or gamma rays	PC-3	+, PD*		[40]
Lovastatin	ACC-MESO-1	+, PD*		[41]
Atorvastatin	PC-3	+, ND		[42]
Pancratistatin	DU145 and LNCaP	+, PD	+ (Caspase dependent)	[43]
Red-Br-nos (ROS inducer)	PC-3	+, PS	+ (Caspase independent)	[44]
Nelfinavir (protease inhibitor)	DU145, PC-3, and LNCaP	–, PD*	+	[45]
BEZ235-PDT	PC-3	+, PS		[46]
EI201 (PI3K/AKT/mTOR pathway inhibitor)	PC-3	+, PS and PD	+	[47]
Arsenic trioxide (ATO)+IR	LNCaP and PC-3	+, PD*	+	[48]
Casodex (anti-androgen)	LNCaP and LAPC4	+, PS*		[49]
Androgen deprivation	LNCaP	+, PS*		[50]
ABT-737 (BH3 domain mimetics)	PC-3 and LNCaP	+, PS		[51]
(−)-Gossypol and sorafenib	PC-3 and DU-145	+, PD* for PC-3 +, PS for DU145	+ for DU-145	[25]
(−)-Gossypol	C4-2B, LNCaP, PC-3, and DU-145	+, PD	+ (Caspase dependent)	[52]
Sabutoclax (apogossypol derivative)	PC-3 and DU-145	+, PS*	+	[53]
Apogossypolone	LNCaP and PC-3	+, PS	+	[54]
Ursolic acid	PC-3	+, PS*		[55]
Docetaxel	DU-145 R, 22RV1 R, and PC-3 R	–, PS		[56]
22 (ILK inhibitor)	PC-3 and LNCaP	+, PD*	+	[57]
Embelin (IAP inhibitor)+IR	PC-3		– (Caspase independent)	[58]

(continued)

Table 19.1 (continued)

Treatments	Cell lines	Autophagy	Apoptosis	Citations
YM155 (survivin inhibitor)	PC-3 and LNCaP	+, PD	+(Caspase dependent)	[59]
Irinotecan	prostate SCC	+, ND		[60]
NPI-0052, bortezomib	LNCaP-Pro5 and PC-3	+, PS		[61]
Saracatinib, PP2 [107] (Src inhibitors)	LNCaP and PC-3	+, PS	+	
Sorafenib	PC-3, DU145, and CWR22Rv1	+, ND	+	[62]
H40, SAHA (HDAC inhibitors)	PC-3 M	+, PD	+	[63]
Cysmethynil (inhibitor of icmt)	PC-3	+, PD*	–	[64]
Soraphen A (acetyl-CoA carboxylase inhibitor)	LNCaP and PC-3 M		+, PS	[65]
MG132 (protease inhibitor)	PC-3	+, PD*	+ (Partial caspase dep)	[66]
Ascorbate	Six prostate cancer cell lines	+, PD	+	[67]
Gamma-tocotrienol	PC-3 and LNCaP	+, PD	+	[68]
Fisetin	PC-3, DU145, and LNCaP	+, PD*		[69]
Zoledronate	PC-3	+, ND	–	[70]
Zoledronic acid	PC-3, DU145, and LNCaP	+, PD	+ (Caspase dependent)	[71]
Phenethyl isothiocyanate (PEITC)	TRAMP mice	+, ND		[72]
	LNCaP and PC-3	+, PD	+	[73]
	LNCaP, PC-3, and PrEC	+, PD*	+	[74]
Rad001 + propachlor	PC-3 and C4-2	+, PD*	+	[75]
Rapamycin	LNCaP, DU145, PC-3, and PrEC	+, ND		[76]
RAD001 (+IR)	PC-3 and DU145	+, PD	+	[24]
DP, DMOG (hypoxia mimetics)	PC-3AR+	+, PD*		[77]
Sphingosine 1-phosphate (S1P)	PC-3	+, PS*		[78]
15d-PGJ2 (thiol reactive cyclopentenone prostaglandin)	DU145		+ (Caspase independent)	[79]
Trptorelix-1 (GnRH-II antagonist)	PC-3	+, PS		[80]
Metformin + 2DG	LNCaP	+, PD –, Metformin alone	+, p53-dependent	[81]
2-Deoxyglucose (2DG)	PC-3 and LNCaP	+, ND		[82]
4-IBP (sigma1 agonist)	PC-3	+, ND	–	[83]

(continued)

Table 19.1 (continued)

Treatments	Cell lines	Autophagy	Apoptosis	Citations
Lysophosphatidic acid (LPA)	PC-3	–, PS		[84]
Thapsigargin	DU145 (Bax-deficient)		+ (Caspase independent)	[85]
A23187, tunicamycin, Thapsigargin, brefeldin	DU145	+, PS		[86]
Sulforaphane (SFN)	PC-3	+, ?	–	[87]
	LNCaP and PC3	+, PS	+	[88]
	PC3 and LNCaP	+, PS*	–	[23]
Biologicals				
rhArg (recombinant human arginase)	LNCaP, PC-3, and DU145	+, PD	–	[89]
Neuregulin (NRG)	LNCaP	+, PD*		[90]
	LNCaP	+, PD*	+ (Caspase independent)	[91]
Poly(I:C) (TLR3 ligand)	LNCaP, PC-3, and DU145	+, PS* and PD*		[92]
IL-24	DU145	+, PS*		[93]
ADI (arginine deiminase)	CWR22Rv1, PC-3, and LNCaP	+, PS* and PD*	+	[13]
CCL2	PC-3, DU145, and C4-2B	+, PS* and PD*		[94]
Physicals				
Ionizing radiation	PC-3MM2 and DU145	+, PS	–	[95]
Photodynamic therapy (PDT)	DU145	+, PD	+	[96]
	DU145	+, PS and PD	–	[97]

Treatments include small molecules, biological and physical methods. "+" and "–" denote up or down regulates autophagy

PS pro-survival, *PD* pro-death, *ND* not determined, and * signifies validation of the pro-survival or pro-death properties of autophagy using genetic (siRNA targeting ATGs) or pharmacological (3-MA, chloroquine, etc.) inhibitors.

direct down modulation of the mTOR/S6K/4EBP pathway with consequential activation of autophagy [104]. P53 induces autophagy by transcriptionally activating a number of autophagy genes (ATGs) and Damage-Regulated-Autophagy Modulator (DRAM) [105, 106]. The mechanisms associated with autophagy suppression are less clear. Docetaxel affects microtubule organization and likely the trafficking of autophagosome components, and thus is inhibitory for autophagy flux. Metformin, used to treat insulin resistance, works by activation of AMPK, which in theory should trigger autophagy. Yet, the outcome is the opposite, due to its added effect on down modulation of Beclin 1, a critical component in the initial formation of the autophagosome [12].

A critical question that follows is whether autophagy induction by these agents counteracts or contributes to the killing effects. If the former is the case, suppressing autophagy should enhance the drug effects. On the other hand, if cell

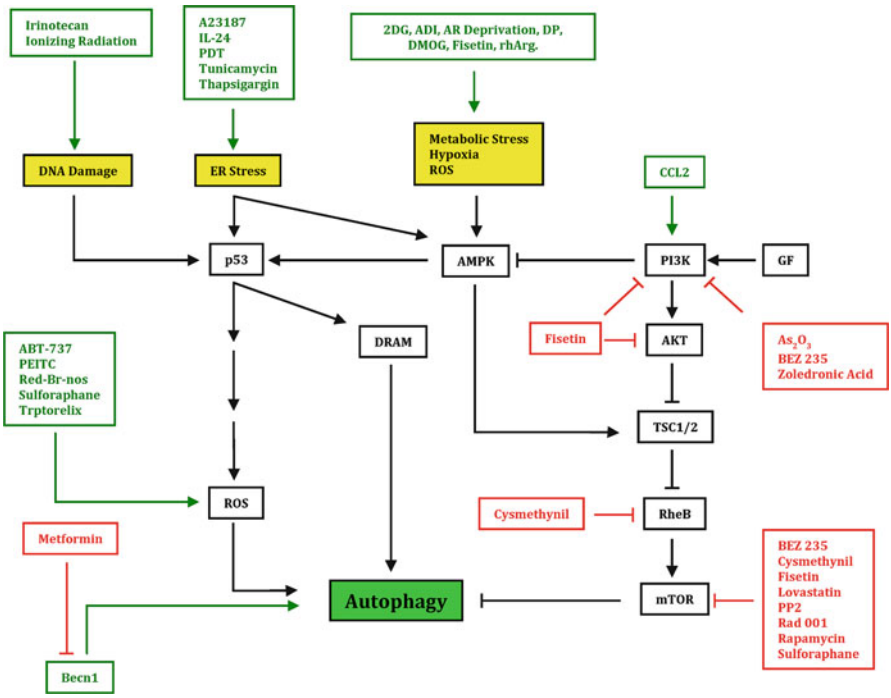


Fig. 19.2 Various types of treatments can cause metabolic stress, ER stress, hypoxia, and ROS, which can then activate the energy sensor AMPK pathway resulting in activation of p53 and/or inhibition of mTOR pathway, ultimately leading to autophagy induction. Alternatively, certain types of treatment can inhibit autophagy by inhibiting components in the previously mentioned pathways. Details about these treatments are listed in Table 19.1 with reference.

death is driven by excessive autophagy (type II programmed cell death), the treatment will be highly valuable in overcoming drug resistance resulting from mutations or aberrations in type I program death pathway (i.e., caspase-dependent apoptosis). A standard way of distinguishing whether autophagy accompanying the treatment counteracts or contributes to cell death is to use genetic or pharmacological inhibitors of autophagy to block the process and to study their effects on cell killing. When this was done (marked as * in Table 19.1), much of the treatment-induced autophagy is pro-survival, i.e., autophagy blockade leads to more cell death. For these cases, autophagy blockers such as chloroquine or hydroxychloroquine will be useful in enhancing the therapeutic effects. The induction of autophagy was found to be the main reason why Src inhibitors only have cytostatic, but not cytotoxic responses, in prostate cancer cells. Addition of chloroquine unleashes the apoptosis-inducing ability of Src inhibitor, resulting in massive cell death [107]. The pro-survival function of autophagy may account for the general failure of most TKIs (tyrosine kinase inhibitor) as monotherapies.

TKIs by virtue of their ability to block mTOR signaling are potent autophagy inducers. Autophagy blockers may, thus, be considered as a general sensitizer for TKIs to overcome apoptosis resistance. The recognition that autophagy may be a significant barrier to the efficacy of traditional therapeutics has led to exploration of agents that block autophagy and, thus, eliminate any pro-survival signal allowing the therapeutic greater cytotoxic efficacy. However, it is important to bear in mind that the current autophagy blockers, chloroquine, hydroxychloroquine, 3-methyladenine, and bafilomycin, all have other effects on cellular metabolism. For instance, chloroquine can induce p53 and ER stress, and at a higher concentration, it intercalates DNA; and its effect on cell killing may not solely reside on autophagy blockade. In experimental models, multiple autophagy blockers including siRNA targeting multiple autophagy components need to be carried out to rigorously demonstrate that the effect is truly due to autophagy blockade. While the outcome of clinical trials involving hydroxychloroquine are anxiously awaited (see below), development of more specific autophagy blockers or modulators is required to improve current prostate cancer therapy. Before such a development, as mentioned above, reagents such as metformin could be used as an alternative agent to chloroquine, recognizing that this diabetes drug also has other cellular effects. It is also noteworthy that clomipramine, an antidepressant, was reported to block autophagy flux with an unclear mechanism [108]. The mechanisms associated with autophagy blockade by these "repurposing" drugs deserve further attention.

While much chemotherapy- or targeted therapy-induced autophagy is pro-survival, a few (e.g., Rad001 (in combination with Propachlor) [75], zoledronic acid [71], Atorvastatin [40], YM155 [59] (−)-gossypol [109]) induce autophagic death. These are generally attributed to excessive autophagy with aberrant upregulation of ATGs and Beclin [75, 110]. As described above, the mechanism of autophagic death is not clear, and several questions remain: Is autophagic death dependent on caspase? If not, is the AIF pathway involved? Are mitochondria involved? Is lysosomal rupture involved? If it is caspase independent, what is the DNase involved in DNA fragmentation? Is ENDO-G involved? Is DNase II (lysosomal DNase) involved? The above examples provide models to delve into the cellular death mechanism triggered by excessive autophagy. That said, in the literature, there are claims of autophagic death, based on the appearance of autophagosomes when cells are dying or on the supposition that death is caspase independent. Neither provides definitive evidence for autophagic death, unless genetic and pharmacological inhibitions were done to demonstrate the effects of autophagy blockade. Indeed, in a recent publication, Shen et al. [111] screened 1400 compounds from the National Cancer Institute library in the presence and absence of autophagy flux inhibitors and concluded that there is little evidence of "autophagic death." Thus, we need desperately a standard metric to define autophagic death and to ascertain its relationship to apoptosis and necrosis. More importantly, we need biomarkers that allow us to discern pro-survival from pro-death autophagy and to provide a practical guide for administering autophagy modulators as an adjunctive therapy.

Autophagy Modulators in Current Clinical Trials

The observation that autophagy is frequently upregulated or induced in prostate cancer cell lines as well as in tumors in response to chemotherapy, radiotherapy, or hormonal therapy have prompted wide interest in using autophagy modulators as novel adjunctive agents to increase the efficacy of cancer therapeutics. Using autophagy inhibitors to block the pro-survival mechanism in these setting may enhance tumor cell death as demonstrated in numerous preclinical studies of xenograft models [107]. Currently, more than 22 Phases I/II clinical trials in the USA are testing the efficacy of autophagy modulators (Table 19.2, based on a query at http://www.clinicaltrials.gov). The most well-studied autophagy inhibitors, metformin, chloroquine (CQ), and the derivative hydroxychloroquine (HCQ), are being tested either alone or in combination with numerous cancer therapeutics including carboplatin, paclitaxel, temsirolimus, and the new mTOR inhibitor, RAD001. Chloroquine blocks autophagy at later stages in the autophagic cascade by interfering with lysosome acidification and impairing autophagosome degradation. Preclinical studies are focusing on earlier stages of autophagy initiation. There are multiple suitable targets in the earlier stages of autophagy induction, including the activation of AMPK or inhibition of PI3K pathway that could be used to block autophagy in future clinical testing. Given the importance of autophagy in cancer therapy, it is to be expected that more specific modulators will be developed to expand the repertoire of agents which target the autophagy pathway.

Concluding Remarks

As summarized in this review, autophagy and cancer therapeutics are now inseparable. The majority of cancer therapies induce autophagy as a stress response and in the majority cases, autophagy is protective for tumor cells, which may account for the general failure of many of these reagents including tyrosine kinase inhibitors as monotherapy. Autophagy blockade using chloroquine or other agents overcomes apoptosis resistance and enhances the therapeutic efficacies. One anxiously awaits the outcome of the ongoing clinical trials using the autophagy modulators. As none of the currently used autophagy inhibitors are specific for the autophagy pathway, it is desirable to develop additional autophagy inhibitors. Although fewer in number, some therapeutic reagents appear to induce excessive autophagy leading to autophagic death. The mechanism of autophagic death remains unclear, although the dying cells morphologically look very differently from apoptosis. It is important to distinguish cells that die “from” autophagy from those that die “with” autophagy by using genetic or pharmacological autophagy inhibitors. Caspase-independent apoptosis may indicate autophagic death, but it is not absolute. An important challenge in the future is to develop biomarkers that can distinguish normal vs. excessive autophagy, and to define the “threshold” over which autophagy becomes pro-death. These

Table 19.2 Ongoing clinical trials with autophagy inhibitors

	Interventions:	Condition	Status	Study
1	HCQ	Breast cancer	Recruiting	Autophagy inhibition using hydrochloroquine in breast cancer patients
2	HCQ, gemcitabine	Advanced adenocarcinoma	Recruiting	A phase I/II/pharmacodynamic study of hydroxychloroquine in combination with gemcitabine/abraxane to inhibit autophagy in pancreatic cancer
3	Paclitaxel, carboplatin, HCQ, bevacizumab	Non-small cell lung cancer	Active, not recruiting	Hydroxychloroquine + carboplatin, paclitaxel, and bevacizumb in non-small cell lung cancer (NSCLC)
4	HCQ	Melanoma (skin)	Recruiting	Hydroxychloroquine in patients with stage III or stage IV melanoma that can be removed by surgery
5	HCQ, RAD001	Metastatic clear cell renal cell carcinoma	Recruiting	Autophagy inhibition to augment mTOR Inhibition: a phase i/ii trial of RAD001 and hydroxychloroquine in patients with previously treated renal cell carcinoma
6	Chloroquine, A-CQ 100	Small cell lung cancer	Not yet recruiting	Chloroquine as an anti-autophagy drug in stage iv small cell lung cancer (SCLC) patients
7	Paclitaxel, carboplatin, HCQ, bevacizumab	Non-small cell lung cancer, recurrent NSCLC	Recruiting	Modulation of autophagy in patients with advanced/recurrent non-small cell lung cancer—phase II
8	HCQ, ixabepilone	Breast cancer	Terminated	Ixabepilone and hydroxychloroquine in treating patients with metastatic breast cancer
9	HCQ, oxaliplatin, 5-fluoro-uracil, bevacizumab	Advance rectal cancer, colon cancer	Recruiting	FOLFOX/bevacizumab/hydroxychloroquine (HCQ) in colorectal cancer
10	HCQ	Renal cell carcinoma	Recruiting	Study of hydroxychloroquine before surgery in patients with primary renal cell carcinoma
11	CQ: (500 mg/week), CQ: (250 mg/week)	Breast cancer	Recruiting	Study of the efficacy of chloroquine in the treatment of ductal carcinoma in situ (The PINC Trial)
12	Docetaxel, HCQ	Prostate cancer	Suspended	Docetaxel and hydroxychloroquine in treating patients with metastatic prostate cancer

(continued)

Table 19.2 (continued)

	Interventions:	Condition	Status	Study
13	Bevacizumab, XELOX regimen, HCQ	Colorectal cancer	Recruiting	Hydroxychloroquine, capecitabine, oxaliplatin, and bevacizumab in treating patients with metastatic colorectal cancer
14	HCQ, sirolimus, vorinostat	Advanced cancers	Recruiting	Sirolimus or vorinostat and hydroxychloroquine in advanced cancer
15	HCQ, vorinostat	Malignant solid tumor	Active, not recruiting	Hydroxychloroquine + vorinostat in advanced solid tumors
16	Bevacizumab, carboplatin, HCQ, paclitaxel	Lung cancer	Terminated	Hydroxychloroquine, carboplatin, paclitaxel, and bevacizumab in treating patients with recurrent advanced non-small cell lung cancer
17	HCQ, erlotinib	Non-small cell lung cancer	Recruiting	Erlotinib with or without hydroxychloroquine in chemo-naive advanced NSCLC and (EGFR) mutations
18	HCQ,imatinib mesylate	Leukemia	Recruiting	Imatinib mesylate with or without hydroxychloroquine in treating patients with chronic myeloid leukemia
19	HCQ,IL-2	Metastatic renal cell carcinoma	Recruiting	Study of hydroxychloroquine and aldesleukin in renal cell carcinoma patients (RCC)
20	HCQ, gemcitabine	Pancreatic cancer	Recruiting	Study of presurgery gemcitabine + hydroxychloroquine (GcHc) in stage IIb or III adenocarcinoma of the pancreas
21	Capecitabine, HCQ, Proton Radiation	Pancreatic cancer	Recruiting	Short course radiation therapy with proton beam capecitabine and hydroxychloroquine for resectable pancreatic cancer
22	HCQ	Pancreatic cancer	Recruiting	Hydroxychloroquine in previously treated patients with metastatic pancreatic cancer

Based on data from ClinicalTrials.gov. http://clinicaltrials.gov/ct2/results?term=autophagy&Search=Search Accessed 5 Oct 2012

studies will provide guides to the application of an autophagy inhibitor as an adjunctive therapy. It is also important to recognize that autophagy and apoptosis regulate each other, sometimes in a positive way, and other times negative. A thorough understanding of the intricate network that connects autophagy and apoptosis holds the key to develop and apply autophagy modulators in cancer therapy.

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