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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.

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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

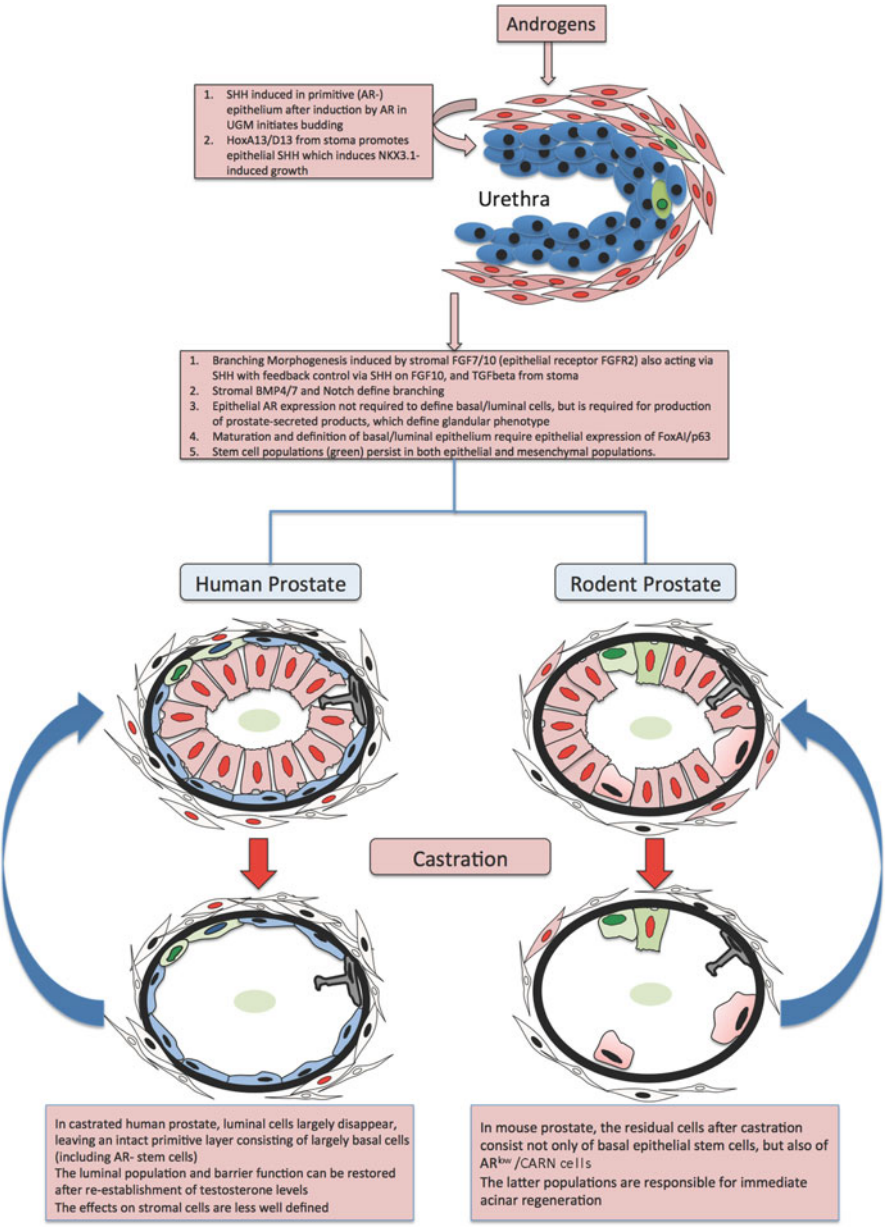

Fig. 1.1 *Stem cell localization in development of normal prostate and after castration-induced prostate gland involution*. In both embryonic and adult prostate glands, stem cells in the epithelial and stromal compartments are shown in *green*, basal cells in *blue*, and luminal cells in *pink*, with 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].