

Primary cell cultures as models of prostate cancer development

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Abstract

This review focuses on primary cultures of human prostatic epithelial cells and their applications as models of normal and malignant biological behavior. Current abilities to culture cells from normal tissues, from premalignant dysplastic lesions (prostatic intraepithelial neoplasia), from primary adenocarcinomas, and from metastases are described. Evidence for representation of the inter-related cells of the normal prostatic epithelium — stem cells, basal epithelial cells, secretory epithelial cells, transit amplifying cells and neuroendocrine cells — in primary cultures is presented. Comparisons between normal and cancer-derived primary cultures are made regarding biological activities relevant to carcinogenesis, such as proliferation, apoptosis, differentiation, senescence, adhesion, migration, invasion, steroid hormone metabolism, other metabolic pathways and angiogenesis. Analyses of tumor suppressor activity, differential gene expression and cytogenetics in primary cultures have revealed changes relevant to prostate cancer progression. Preclinical studies with primary cultures have provided information useful for designing new strategies for chemoprevention, chemotherapy, cytotoxin therapy, radiation therapy, gene therapy and imaging. While the behavior of normal primary cultures is often used as a basis for comparison with established, immortal prostate cancer cell lines, the most informative studies are performed with donor-matched pairs of normal and malignant primary cultures, grown under identical conditions. Challenges that remain to be addressed if the full potential of primary cultures as a model system is to be realized include isolation, culture and characterization of stem cells, improved methodology to induce or maintain a fully differentiated, androgen-responsive phenotype, and identification of cell surface antigens or other markers with which to purify pure populations of live cancer or premalignant cells apart from non-malignant epithelial cells prior to culture.

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Introduction

Historically, *in vitro* cultures of human prostatic cells have been limited in availability and scope compared with those from many other organs. Three spontaneously established cell lines, PC-3, DU 145 and LNCaP, are by far the most commonly used cell culture models of prostate cancer (Bosland *et al.* 1996). Although other prostate cancer cell lines are available, recent studies revealed that many of these are in fact derivatives of the three aforementioned cell lines or other non-prostatic lines (van Bokhoven *et al.* 2003). The few bona fide cell lines, almost all derived from metastases, do not span the range of prostate cancer phenotypes, and in particular are not representative of primary adenocarcinomas of the prostate. Furthermore, the

question of how extensively long-term culture alters the biological properties of cell lines is always lurking. For these and other reasons, primary cultures of malignant prostatic cells and their normal epithelial counterparts are sought.

During the past 20 years, many of the technical hurdles involved in growing primary cultures of human prostatic epithelial cells have been overcome, and a variety of more or less similar methods have been reported (for review see Peehl & Sellers 2002). Most primary cultures are derived from radical prostatectomy specimens, but methods for culturing cells from needle biopsies, from fluid obtained by prostatic massage, and from bone marrow aspirates have been described. The use of primary cultures is becoming

more widespread, especially with the advent of commercial availability of normal cells, and they have been used in many diverse studies. The growing body of information about the molecular and cellular features of prostate tissues has permitted more extensive and definitive characterization of primary cultures. This article will review the characteristics of primary cultures of human prostatic epithelial cells, with special emphasis on their ability to model processes involved in prostatic carcinogenesis. Information obtained from primary cultures derived from benign prostatic hyperplasia (BPH) will not for the most part be included in this review. For a discussion of the molecular and cellular biology of BPH and the properties of primary cultures derived from BPH tissues, the reader is referred to a recent review (Lee & Peehl 2004). Although generation of primary cultures of mouse prostatic cells is becoming more frequent, this article also will not include any information about rodent cell culture models. Finally, although cell lines created by introduction of immortalizing genes into primary cultures are often used as representative of the normal or cancer cells of origin (for reviews see Webber *et al.* 1996, 1997), studies with these cells also will not be included here because the relevance of these lines to normal or cancer biology is controversial.

Normal prostatic epithelial cells

The normal prostatic epithelium is composed of five inter-related cell types — stem cells, basal epithelial cells, transit amplifying cells, neuroendocrine cells and secretory epithelial cells. The ability of these cell types to be maintained *in vitro* is discussed below.

Basal epithelial cells

Basal cells form a single layer on the basement membrane underlying the normal prostatic epithelium. Classic markers of basal epithelial cells of normal glands of the human prostate are cytokeratins 5 and 14 (Brawer *et al.* 1985). Most investigators report that almost every cell in primary cultures derived from normal tissues expresses these basal cytokeratins (Brawer *et al.* 1986, Gao *et al.* 2001). Proliferation is also confined to the basal cells of normal tissues (McNeal *et al.* 1995, Kyprianou *et al.* 1996) and, similarly, primary cultures of normal prostatic epithelial cells are proliferative.

In 1994, a list of genes whose expression had been localized to basal cells of normal prostatic tissues up to that time was published (Ware 1994). More recently,

Liu *et al.* (2002) prepared a cDNA expression library from basal cells purified directly from enzymatically digested tissues and identified genes expressed by these cells. Of these basal-associated genes, only a subset has been evaluated in cultured cells. One of the basal-associated genes expressed by primary cultures of normal prostatic epithelial cells is the transcription factor ETS-2 (Liu *et al.* 1997a). ETS-2 positively regulates the expression of maspin, a protease inhibitor that is also found in basal cells of prostatic tissues and in cultured normal cells (Zhang *et al.* 1997). Maspin has gained attention as a putative tumor suppressor gene (Sheng *et al.* 1996), and microarray analyses have pinpointed maspin as a gene differentially expressed between non-malignant and prostate cancer tissues (Chen *et al.* 2003).

B cell translocation gene 2 (BTG2), a p53 target gene that negatively regulates cell cycle progression, is also expressed in basal cells of prostatic glands and in primary cultures from normal tissues, reaching its highest level in quiescent cells (Ficazzola *et al.* 2001). BTG2 is absent in premalignant dysplastic glands and in prostate cancer, perhaps related to loss of growth control in these entities. Other proteins localized to the basal epithelia of prostatic glands and expressed by primary cultures of normal prostatic epithelial cells include the growth factor receptor ErbB1 (Adam *et al.* 1999), CD44 (Liu *et al.* 1997b), and the estrogen receptor (ER)- β (Leav *et al.* 2001, Pasquali *et al.* 2001).

Basal epithelial cells *in vitro* should theoretically make a basement membrane, but few studies have investigated the production of basement membrane or other extracellular matrix proteins in detail. Cussenot *et al.* (1994) reported that cultures of normal prostatic epithelial cells did not make the basement membrane protein, type IV collagen. Cultured prostatic epithelial cells aberrantly make the mesenchymal-associated extracellular matrix protein, vimentin, but this is true of most cultured epithelial cells and is apparently a common artifact of *in vitro* propagation (Sherwood *et al.* 1989, Kooistra *et al.* 1995, Goossens *et al.* 2002).

Overall, many features of primary cultures of epithelial cells derived from normal prostatic tissues are consistent with a basal-like phenotype — proliferative ability, expression of cytokeratins 5 and 14, and expression of other basal-associated genes (Table 1).

Secretory epithelial cells

Differentiated secretory epithelial cells line the lumens of normal prostatic glands. Whereas basal cells express cytokeratins 5 and 14, secretory cells express cytokeratins 8 and 18 (Brawer *et al.* 1985). A subpopulation of

Table 1 Basal cell markers expressed by primary cultures of normal prostatic epithelial cells

Marker	Reference
Cytokeratin 5	Brawer <i>et al.</i> (1986), Gao <i>et al.</i> (2001)
Cytokeratin 14	Brawer <i>et al.</i> (1986), Gao <i>et al.</i> (2001)
ETS-2	Liu <i>et al.</i> (1997a), Liu & Peehl (2001)
Maspin	Zhang <i>et al.</i> (1997)
BTG2	Ficazzola <i>et al.</i> (2001)
ErbB1	Adam <i>et al.</i> (1999)
CD44	Liu <i>et al.</i> (1997b), Liu & Peehl (2001)
ER β	Leav <i>et al.</i> (2001), Davis <i>et al.</i> (2002)
p63	Signoretti <i>et al.</i> (2000), Davis <i>et al.</i> (2002)
c-Met	You <i>et al.</i> (2003)
17 β -HSD	He <i>et al.</i> (2003)
Epithelial glycoprotein	Liu & Peehl (2001)
Collagenase	Liu & Peehl (2001)
Zinc- α_2 -glycoprotein	Liu & Peehl (2001)
Fibronectin receptor- β	Liu & Peehl (2001)
SEF2	Liu & Peehl (2001)
CD49b	Liu & Peehl (2001)
CD49f	Liu & Peehl (2001)
CD55	Liu & Peehl (2001)
CD59	Liu & Peehl (2001)
CD95	Liu & Peehl (2001)
CD104	Liu & Peehl (2001)

cells in normal primary cultures expresses cytokeratins 8 and 18 (Brawer *et al.* 1986, van Leenders *et al.* 2000, Gao *et al.* 2001, Goossens *et al.* 2002). However, since these same cells simultaneously express the basal cell cytokeratins 5 and 14, this pattern of cytokeratin expression suggests lack of complete differentiation. Cells in the prostatic epithelium that express markers of both basal and secretory cells are referred to as intermediate cells or transit amplifying cells (Bonkhoff *et al.* 1994), and their significance is discussed in a forthcoming section. Cytokeratin 19, expressed by both basal and secretory cells of the prostatic epithelium, is also expressed by cultured cells (Peehl *et al.* 1996, Robinson *et al.* 1998).

15-Lipoxygenase (LOX)-2 is a secretory cell-specific enzyme (Shappell *et al.* 1999) that is expressed in primary cultures of normal prostatic cells (Tang *et al.* 2002). Decreased expression of 15-LOX-2 in prostate cancer suggests tumor suppressor functions (Jack *et al.* 2000), and this concept is further supported by anti-proliferative effects of 15-LOX-2 metabolites on normal prostatic epithelial cells (Tang *et al.* 2002).

A hallmark of prostatic secretory cells is the expression of androgen receptor (AR) and prostate-specific antigen (PSA) (Leav *et al.* 1996). Most investigators report that normal primary cell cultures express low or no AR or PSA and do not respond to androgen or respond minimally (for review see Peehl & Sellers 2002). Methylation of the gene promoter has been ruled out as the mechanism responsible for lack of expression of AR in normal primary cultures, and in fact AR mRNA can be found in these cells (Grant *et al.* 1996, Jarrard *et al.* 1998, Tekur *et al.* 2001). This phenotype is reminiscent of basal epithelial cells in the prostate, in which AR transcripts are present but are not translated into protein (Leav *et al.* 1996). Therefore, lack of robust expression of AR and PSA is another feature of primary cultures that more resembles a basal than a secretory cell phenotype. However, despite the absence of AR protein, primary cultures are proving useful for studies of AR coregulators. One of these, ARA70, was noted to be expressed at high levels in normal primary cultures compared with prostate cancer cell lines (Tekur *et al.* 2001).

Although investigators have attempted to achieve a fully differentiated secretory phenotype of prostate cells *in vitro*, success has been limited. While retinoic acid increases levels of the secretory cell cytokeratins 8 and 18 in primary cultures, the cells continue to express basal cell cytokeratins, and other differentiation markers such as PSA remain low (Peehl *et al.* 1994b, Goossens *et al.* 2002). Continuous exposure to collagenase was reported to retain androgen response (Mitchen *et al.* 1997), but this methodology has not been tested further. Culture in medium containing fibroblast growth factor (FGF)-7 was said to increase AR mRNA levels in normal cells, but protein was not measured (Planz *et al.* 2001).

Modest success at promoting differentiation has been achieved by three-dimensional culture in Matrigel (Fong *et al.* 1991, Hudson *et al.* 2000). Epithelial cells grown on collagen gels containing stromal cells also were reported to express PSA and another differentiation marker, prostate-specific membrane antigen (PSMA) (Hall *et al.* 2002). A recent report by Lang *et al.* (2001) revealed that serum may be the secret ingredient that has been missing in culture media used to promote differentiation. In three-dimensional cultures in Matrigel in the absence of serum and stromal cells, solid masses of cellular spheres formed from normal prostatic epithelial cells isolated directly from tissues or after short-term culture. The addition of 2% serum induced formation of a lumen surrounded by one or two layers of cuboidal cells, and cytokeratins 5, 14, and 18 were variably expressed. PSA and AR were

weakly expressed. Addition of stromal cells, androgen and estrogen induced polarization of the epithelium. Interestingly, these polarized structures could be formed only by CD44+ (basal) cells selected from the original tissue digests, while CD44- cells were unable to create these structures. While exact reproduction of basal and secretory cell phenotypes was not achieved, this appears to be a significant step forward.

As another approach to study secretory cells in culture, Liu *et al.* (1997b) isolated almost pure populations of live basal and secretory cells from enzymatically digested tissues, taking advantage of differential expression of CD44 and CD57 cell surface antigens by basal and secretory cells respectively. After being sorted by flow cytometry, the separated cell populations were cultured overnight and characterized. The cell populations continued to express many of the genes of the basal or secretory cell transcriptomes that these investigators had previously identified. However, expression of PSA was unexpectedly absent from CD57+ cells, despite the continued expression of other secretory cell genes. Only when the secretory cells were co-cultured with prostatic stromal cells was PSA expression maintained. Interestingly, stromal cells also induced PSA expression in CD57- (basal) cells. Altogether, these results add to the overall impression that stromal-epithelial interactions are indeed critical for prostatic epithelial cell differentiation, and that secretory cells are derived from a basal cell precursor.

Secretory cell markers that are expressed by primary cultures of normal prostatic epithelial cells are summarized in Table 2.

Neuroendocrine cells

Neuroendocrine cells make up a small percentage of the normal prostatic epithelium (Bonkhoff 1998). A variety of evidence points to the origin of these neuroendocrine cells from a pluripotent (stem?) cell that also generates secretory cells in the prostate (Bonkhoff 1998). The biological role of neuroendocrine cells in the prostate is not clear, but it has been hypothesized that the cytokines secreted by these cells influence growth or differentiation of surrounding epithelial cells (Abrahamsson 1999). Neuroendocrine differentiation in primary cultures has not been widely investigated, but Rumpold *et al.* (2002) reported the presence of non-proliferating cells that coexpressed cytokeratins 5, 14 and 18, as well as the neuroendocrine marker chromogranin A, in primary cultures of prostatic epithelial cells. Table 3 lists neuroendocrine markers expressed in primary cultures.

Table 2 Secretory (luminal) cell markers expressed by primary cultures of normal prostatic epithelial cells

Marker	Reference
Cytokeratin 8	Brawer <i>et al.</i> (1985), van Leenders <i>et al.</i> (2000), Gao <i>et al.</i> (2001), Goosens <i>et al.</i> (2002)
Cytokeratin 18	Brawer <i>et al.</i> 1985, van Leenders <i>et al.</i> (2000), Gao <i>et al.</i> (2001), Goosens <i>et al.</i> (2002)
15-LOX-2	Tang <i>et al.</i> (2002)
AR (+/-)	Reviewed in Peehl & Sellers (2002)
PSA (+/-)	Reviewed in Peehl & Sellers (2002)
ARA70	Tekur <i>et al.</i> (2001)
PSMA (+/-)	Hall <i>et al.</i> (2002)
CD9	Liu & Peehl (2001)
CD24	Liu & Peehl (2001)
CD10	Liu & Peehl (2001)
CD13	Liu & Peehl (2001)
CD26	Liu & Peehl (2001)
CD107a	Liu & Peehl (2001)
CD117	Liu & Peehl (2001)

Transit amplifying cells

Simultaneous expression of basal cell cytokeratins 5 and/or 14 and secretory cell cytokeratins 8 and/or 18, as occurs in a subset of cells in primary cultures, is also seen in a small number of cells in the prostatic epithelium (Bonkhoff *et al.* 1994). Such cells have been referred to as 'transit amplifying cells' or 'intermediate' cells. These proliferative cells express features of basal and secretory and/or neuroendocrine cells, and combinations thereof. It has been proposed that these transit amplifying cells are stem-like cells or progenitor cells that are in the process of generating differentiated cell populations but have not yet completely committed to one particular lineage. The phenotype of primary cultures — proliferative and simultaneously expressing markers of both basal and secretory cells, and possibly neuroendocrine cells — most resembles that of transit amplifying cells (for review see Uzgaré *et al.* 2004). For example, Tran *et al.* (2002) described a subpopulation in primary cultures of normal prostatic epithelial cells that coexpressed

Table 3 Neuroendocrine markers expressed by primary cultures of normal prostatic epithelial cells

Marker	Reference
Chromogranin A	Rumpold <i>et al.</i> (2002)
Neuron-specific enolase	Liu & Peehl (2001)

prostate stem cell antigen and CD44 (basal markers), basal and secretory cell cytokeratins, and AR and PSA (secretory markers). These cells did not express the basal marker, p63. These investigators interpreted their findings as evidence of an intermediate population of cells in transition from a basal to a terminally differentiated secretory phenotype. Garraway *et al.* (2003) provided a similarly detailed analysis of intermediate cells in primary cultures.

Analysis of CD antigens also suggested an intermediate phenotype of cultured prostatic epithelial cells (Liu & Peehl 2001). Liu & True (2002) cataloged expression of CD antigens by constituent cell types of the prostate and identified CD antigens on secretory cells and on basal cells. Normal primary cultures expressed a number of CD antigens in common with basal epithelial cells, but also expressed many CD antigens found on secretory cells, again demonstrating an intermediate phenotype of cultured cells.

The most comprehensive view of the basal- vs secretory-like phenotype of cultured normal prostatic epithelial cells comes from a comparison of basal and secretory cell transcriptome sets to gene sequences in a cDNA library made from cultured normal cells (Liu *et al.* 2002). There was a higher representation of basal cell genes (12.3%) than secretory cell genes (6.7%), showing again the intermediate nature of the cells but suggesting that, overall, the cells are more basaloid than secretory.

Stem cells

Schalken & van Leenders (2003) recently discussed current views regarding stem cells in the prostate. Prostatic stem cells have not been definitively identified in tissues or in culture. A recent study provided evidence that stem cells in the mouse prostate are located proximally (Tsujiura *et al.* 2002), but whether this is true in the human prostate is unknown. It is believed that stem cells reside in the basal epithelial layer. In primary cultures of human prostatic epithelial cells established by Hudson *et al.* (2000), two types of clones were observed. One type was described as small and composed of enlarged, senescent-like cells. The other type was typically large and composed of numerous small, proliferating cells. These investigators proposed that the large proliferative colonies developed from stem cells, but other interpretations are possible.

Expression of $\alpha_2\beta_1$ -integrin is linked to epithelial stem cells. This integrin was used as a marker for sorting putative stem cells from the human prostate and culturing for evaluation (Collins *et al.* 2001). First,

expression of $\alpha_2\beta_1$ -integrin in prostatic epithelium in tissues was evaluated, and about 1% of basal cells highly expressed $\alpha_2\beta_1$ -integrins. Selection of cells from digested tissues that adhered to type I collagen resulted in a cell population that highly expressed α_2 -, α_3 - and α_6 -integrins. Rapidly adhering cells had a high colony-forming efficiency and a basal-like phenotype (cytokeratin 5/14+, PSA-, small subset cytokeratin 18+). Three types of colonies formed: those that did not grow for about a week, then proliferated and formed large colonies, those that grew rapidly from the onset, and those that formed small terminal colonies. Cells that were selected by the ability to attach to type I collagen within 5 min and were then combined with stromal cells and injected into mice occasionally formed epithelial structures with both basal and secretory (PSA/AR+) cells, considered as evidence of stem cell activity.

Expression of telomerase is associated with stem cells, which have extended proliferative ability and are long-lived if in fact not immortal. Therefore, the presence of telomerase in primary cultures is suggestive of the presence of stem cells. Low but detectable telomerase activity in normal prostatic primary cultures has been reported by some investigators (Belair *et al.* 1997, Soda *et al.* 2000), corresponding with evidence of telomerase in basal cells of the normal prostatic epithelium (Bettendorf *et al.* 2003).

It is likely, if stem cells exist in primary cultures established and maintained under standard conditions, that they are lost with serial passage or overgrown by non-stem cells. Gao *et al.* (2001) found that organoids of prostatic epithelial cells, digested from normal tissues and immediately placed in Matrigel and injected s.c. into mice, exhibited stem cell-like activity in that they formed acini with basal and secretory cells in an androgen-dependent manner. But if cells were isolated and grown in primary culture prior to implantation in mice, they remained as small nests of basal cells. Unlike the organoids, the previously cultured cells did not induce infiltration and smooth muscle differentiation of mesenchymal cells, an event that may be critical for maintenance and function of stem cells.

Putative markers of stem cells expressed by primary cultures are summarized in Table 4.

Table 4 Putative markers of stem cells expressed by primary cultures of normal prostatic epithelial cells

Marker	Reference
$\alpha_2\beta_1$ integrin	Collins <i>et al.</i> (2001)
Telomerase	Belair <i>et al.</i> (1997), Soda <i>et al.</i> (2000)

Biological activities of normal primary cultures

Proliferation

Normal prostatic epithelial cells are typically grown in serum-free medium and reported doubling times range from 16 to 36 h (for review see Peehl & Sellers 2002). While this rate of proliferation is much more rapid than that of epithelia in the prostate itself (with a turnover time of ~ 2 years), proliferation can be modulated by reducing the concentrations of mitogens or other components in the medium to attain a more physiological rate of replication (Cussenot *et al.* 1994).

Several previous reviews have discussed growth regulatory factors for prostatic epithelial cells in depth (Byrne *et al.* 1996, Culig *et al.* 1996, Peehl 1996, Ittman & Mansukkani 1997, Djakiew 2000), so a detailed presentation will not be given here. Mitogens for normal prostatic epithelial cells are similar to those for other types of epithelial cells, and include members of the epidermal growth factor (EGF) family, FGF family, nerve growth factor family and insulin-like growth factors (IGFs). Growth-inhibitory factors include transforming growth factor (TGF)- β , vitamin D, and retinoids. Studies with primary cultures have shown that many growth factors are secreted by normal prostatic epithelial cells, and either exert autocrine effects if the cognate receptor is expressed by the epithelial cells, or mediate paracrine effects on cognate receptors present in prostatic stromal cells. Similarly, many growth factors have been identified that are secreted by stromal cells and exert paracrine effects on the epithelium. Primary cultures have not yet revealed factors that mediate stromal–epithelial interactions induced by androgen (andromedins), or factors that maintain homeostasis between basal and secretory epithelial cells.

Recently, additional growth factor receptors and their ligands have been studied in normal prostatic epithelial cells. Receptors for colony-stimulating factor (CSF)-1 were expressed, albeit at relatively low levels (Ide *et al.* 2002). Parallel studies of mouse prostate development demonstrated epithelial expression of CSF-1R during the protrusion of prostatic buds from the urogenital sinus and during the prepubertal and androgen-driven proliferative expansion and branching of the glands, suggesting a developmental role for this tyrosine kinase. Immunohistochemical analysis of CSF-1R in the adult human prostate showed low levels of expression in normal epithelia, with a trend towards increased expression in dysplasia and cancer. Further studies may more definitively

point to a role for CSF-1R in prostate cancer progression.

Another receptor of recent interest is the peroxisome proliferator-activated receptor (PPAR)- γ , a member of the nuclear hormone receptor superfamily. Agonists of PPAR γ have shown some clinical activity in pilot studies on prostate cancer (Koeffler 2003). Treatment of normal prostatic epithelial cells with the PPAR γ agonist, rosiglitazone, resulted in growth suppression and induction of a unique phenotype whose characteristics remain to be more fully elucidated (Xu *et al.* 2003).

Apoptosis

Similar to the low rate of proliferation in the normal prostatic epithelium, apoptosis occurs in less than 1% of the homeostatic epithelium (Kyprianou *et al.* 1996). Many studies suggest that primary cultures of normal prostatic epithelial cells are relatively resistant to the induction of apoptosis. For example, normal cells are resistant to apoptosis-inducing effects of tumor necrosis factor (TNF)- α , despite the presence of TNF- α receptors (Subbarayan *et al.* 2001, Chopra *et al.* 2004). In studies of resistant normal primary cultures and resistant prostate cancer cell lines vs responsive cell lines, resistance to apoptosis correlated with activation of IKK- α and translocation of NF- κ B to the nucleus.

TGF β has been implicated as an apoptotic factor in the prostatic epithelium. TGF β increases after androgen ablation, and treatment with TGF β partially but not entirely induces epithelial apoptosis to the same extent as that caused by deprivation of androgen (Martikainen *et al.* 1990). Nevertheless, in primary cultures of normal prostatic epithelial cells, TGF β typically induces growth arrest and other phenotypic changes, but not apoptosis (Peehl *et al.* 1994b). However, Sutkowski *et al.* (1992) reported that TGF β induced apoptosis in primary cultures if the potent mitogen and survival factor, EGF, was deleted from the culture medium. This finding suggests that the activity of TGF β and/or the ability to undergo apoptosis in general depends on the local milieu of growth and survival factors.

One exception to overall resistance to apoptosis is the sensitivity of normal prostatic epithelial cells to TNF-related apoptosis-inducing ligand (TRAIL), a pro-apoptotic cytokine at one time believed to selectively kill cancer cells without harming normal ones. However, normal prostatic epithelial cells are very sensitive to induction of apoptosis by TRAIL (Nesterov *et al.* 2002). Interestingly, these cells have comparable levels of TRAIL receptors (DR4 and

DR5) to some other types of cells that are resistant to TRAIL. The unusual sensitivity may instead be due to deficiency in anti-apoptotic decoy receptors (DcR1 and DcR2).

Another condition that reportedly causes apoptosis of normal prostatic epithelial cells is deprivation of growth factors (Tang *et al.* 1998). Prostate cancer cell lines and primary cultures of cancer cells were seemingly much more resistant to this deprivation. The differential response was attributed to upregulation of p53 and pro-apoptotic proteins Bax/Bad/Bak, concurrent with downregulation of p21 and low levels of bcl-2, in the normal cells. Additional discussion of apoptosis in normal vs malignant prostatic epithelial cells can be seen in a forthcoming section.

Senescence

Cellular senescence is proposed to be a growth-limiting block that cells must overcome in order to become tumorigenic. It has been suggested that induction of senescence in cancer cells may be an effective therapeutic strategy (Berns 2002). Primary cultures have been used to delineate molecular mechanisms of senescence in prostate cells. Epithelial cells cultured from normal tissues are mortal and are capable of about 30 population doublings before undergoing replicative senescence (Iype *et al.* 1998, Schwarze *et al.* 2001, Peehl & Sellers 2002), although one group described extended culture of normal cells (~50 population doublings) by continuous treatment with interstitial collagenase (Mitche *et al.* 1997). In the early 1990s, Merchant (1990) described cells grown by his methods as going into crisis, then becoming rapidly proliferating. However, this phenomenon has not been described by any other investigator with one exception (Sinisi *et al.* 2002). It should be noted that, until recently, most normal cells were derived from men ~60 years of age or older, undergoing radical prostatectomy or other surgical procedures. Although normal prostatic epithelial cells from young men are now commercially available, a detailed examination of their lifespan in culture has not been reported, and it is possible that they are longer-lived.

The characteristics of serially passaged normal prostatic epithelial cells approaching and undergoing senescence have been described. Increased expression and phosphorylation of p16^{INK4a} accompanies replicative senescence (Sandhu *et al.* 2000), and Schwarze *et al.* (2001) found that alterations in either p16^{INK4a} or pRB are necessary in order for prostatic epithelial cells to bypass senescence. Recent studies further defined the role of cyclin-dependent kinase inhibitors in the

growth arrest that occurs at senescence of prostatic epithelial cells (Schwarze *et al.* 2001). While p16^{INK4a} increased, other proteins including pRB, cyclin D, p19^{INK4d}, and p27^{KIP1} declined. A phase of pre-senescent growth arrest was identified that was accompanied by elevation of Cdk2-associated activity, and transient elevation of p53, p21^{CIP1}, and p15^{INK4b}. A noteworthy finding in this study was that p57^{KIP2} levels increased in senescent human uroepithelial cells but not in prostatic cells, suggesting cell-specific molecular regulation of senescence. Another interesting point is that senescence of prostatic epithelial cells (and urothelium) occurs by telomere-independent mechanisms (Belair *et al.* 1997). Microarray analysis has led to a genetic profile of senescent normal human prostatic epithelial cells (Schwarze *et al.* 2002). Secretion of interleukin (IL)-1 α and IL-8 is also associated with senescent prostatic epithelial cells (Castro *et al.* 2004).

Interferons (IFNs), a family of cytokines, have been implicated in senescence of prostatic epithelial cells. Serial analysis of gene expression revealed that the expression of a set of IFN-activated genes is upregulated during the onset of senescence (Untergasser *et al.* 2002), and Xin *et al.* (2003) recently showed that IFI 16, a member of the IFN-inducible p200-protein family, contributes to senescence of normal prostate cells. These investigators also observed that prostate cancer cell lines either did not express IFI 16 or expressed a variant form, and that overexpression of IFI 16 inhibited growth of cancer cell lines and induced a senescent-like phenotype. It is also interesting to note that loss of expression of IFN-activatable genes correlated with development of cancer in an experimental model of prostate tumor progression (Shou *et al.* 2002). Studies such as these provide insight into the role of senescence as a tumor suppressor.

Whether senescence actually occurs in the human prostate is an interesting question. Two groups have reported the presence of prostatic epithelial cells expressing senescence-associated β -galactosidase, but only in prostates with abundant BPH (Choi *et al.* 2000, Castro *et al.* 2003). The role of senescence in physiological and pathological processes remains an important topic of study.

Adhesion, migration and invasion

Cell motility and migration are essential for morphogenesis of the ductal system of the prostate, and for continuous regeneration of the epithelium in the adult. Increased migration and invasion and decreased adhesion are believed to be instrumental in cancer

progression. Therefore, these activities have been studied in normal cells to better understand physiological processes as well as the impact of changes that occur in cancer.

Migration of normal prostatic cells in culture is very dependent on the composition of the culture medium. For example, in medium containing EGF, cells are very migratory and form colonies consisting of widely spread cells. In contrast, in medium without EGF or with FGF-7 replacing EGF, cells remain in coherent islands with extensive cellular adhesions (Peehl & Rubin 1995). Hepatocyte growth factor (HGF), secreted by prostatic stromal cells, is a potent stimulator of migration and invasion of normal prostatic cells in culture (You *et al.* 2003). The cellular receptor of HGF, c-Met, is known to be present on basal epithelial cells in the prostate, and HGF is believed to regulate branching morphogenesis via paracrine mechanisms (Zhang & Vande Woude 2003). It is interesting that c-Met has also been localized to transit amplifying cells in the prostate, while c-Met is absent on secretory cells, suggesting a need for migration of basal and transit but not fully differentiated cells (van Leenders *et al.* 2002). c-Met is expressed as well on prostate cancer cells, and HGF and overexpression of c-Met have been implicated in metastatic potential (Humphrey *et al.* 1995, Pisters *et al.* 1995, Zhu & Humphrey 2000). An adenovirus expressing a c-Met ribozyme was shown to inhibit growth and metastasis of a prostate cancer xenograft model, further showing the important role of this receptor in cancer (Kim *et al.* 2003).

The membrane-anchored serine protease prostasin is believed to suppress invasion. Expressed in normal prostatic epithelia but downregulated in high-grade cancer, prostasin is expressed by primary cultures of normal cells but not by several of the prostate cancer cell lines (Chen *et al.* 2004). Hypermethylation of the promoter sequence of the gene is responsible at least in part for lower expression in cancer cells. In contrast to high expression of the invasion suppressor, prostasin, in normal cells, MUC18, a cell adhesion molecule associated with metastatic ability, is expressed at low levels in normal prostatic cell cultures but at high levels in several prostate cancer cell lines (Wu *et al.* 2001).

Tumor suppressors

The most widely studied tumor suppressor is p53 (Balint & Vousden 2001). As in many other types of normal human epithelial cells (Flatt *et al.* 1998, Meyer *et al.* 1999), p53 activation by DNA-damaging agents is attenuated in primary cultures of normal prostatic

epithelial cells (Girinsky *et al.* 1995, Bromfield *et al.* 2003). This attenuation has been attributed to a need for progenitor or stem-like cells to repress p53 activity so as not to self-eliminate through p53-mediated apoptosis (Dumble *et al.* 2001). One group has reported elevation of p53 and apoptosis in normal prostatic epithelial cells upon growth factor withdrawal, which did not occur in primary cultures from cancer (Tang *et al.* 1998). Further discussion of p53 in relation to prevention and therapy of prostate cancer is included in forthcoming sections.

The p53 homolog, p63, is present in the basal epithelium of the prostate and in primary cell cultures from normal tissues, which express predominantly the $\Delta Np63\alpha$ isotype (Signoretti *et al.* 2000, Davis *et al.* 2002). The $\Delta Np63\alpha$ isotype acts as a p53-dominant-negative, raising the possibility that expression of this p63 isotype is related to the attenuated activity of p53 in normal cells. Interestingly, p63 expression disappears in senescent cultures of prostatic epithelial cells (Davis *et al.* 2002), at a time when p53 increases (see the discussion of senescence above). For the most part, p63 expression is absent in prostate cancer (Signoretti *et al.* 2000, Davis *et al.* 2002), although expression in primary cultures from tumors has not been determined. A role in stem cell functions has been attributed to p63 (McKeon 2004), but its functions in prostatic epithelial cells are undefined.

Another gene, DOC-2/DAB2, has tumor suppressor activity in prostate cancer cell lines, and studies with normal prostatic epithelial cells have suggested that this protein acts by binding to and inactivating c-Src, which is involved in the Ras-mitogen-activated protein kinase pathway (Zhou *et al.* 2003).

Dysplasia

Dysplasia, also known as prostatic intraepithelial neoplasia, is considered to be a premalignant lesion in the prostate (McNeal 1988, Bostwick *et al.* 1996). Histologically, dysplasia is typified by enlarged nuclei, attenuation of the basal epithelial cell layer, proliferation of secretory cells, and aberrant differentiation. Since no distinctive marker of dysplastic cells has been recognized, culture of dysplastic epithelial cells would depend on the ability to grossly dissect tissues composed almost entirely of dysplastic epithelia, and this is virtually impossible. Until a method to select live dysplastic cells apart from normal or cancer cells is devised, probably based on the identification of dysplasia-specific cell surface proteins, primary culture of validated populations of dysplastic cells is unlikely.

Primary adenocarcinomas

Primary cultures of malignant epithelial cells are typically established from primary adenocarcinomas of the prostate using methodology similar to that used to culture normal cells (for review see Peehl & Sellers 2002). Because no cancer-specific cell surface antigen has been identified that can be used to sort live cells, establishment of primary cultures of cancer cells depends on precise and detailed histopathological examination of the tissues of origin to exclude the presence of non-malignant epithelial cells. In a number of laboratories, primary cultures have been prepared from well-differentiated cancers (Gleason grade 3) as well as from poorly differentiated cancers (Gleason grades 4 and/or 5). Most investigators avoid establishment of primary cultures from individuals undergoing androgen-deprivation therapy, to avoid unknown effects on cellular biology. The following sections describe the characteristics of cancer-derived primary cultures in comparison with primary cultures from normal tissues and immortal, established cancer cell lines.

Basal vs secretory phenotype of primary cultures from cancer

A distinctive feature of prostatic adenocarcinomas is the absence of basal epithelial cells. While this is usually simply noted as a histopathological feature, it is interesting to consider whether the absence of basal cells might not in fact be a significant biological component of malignant behavior. A role for basal cells in the regulation and suppression of prostate cancer cells was suggested from work carried out with primary cultures (Miniati *et al.* 1996). When a prostate cancer cell line was grown on matrix derived from basal-like normal cells, growth was suppressed and the pattern of transcription was altered. It is notable that the basal cell layer becomes attenuated in the premalignant lesion, dysplasia, finally disappearing completely at the point of frank invasion (McNeal *et al.* 1991). Therefore, the disappearance of the basal layer and its possible tumor suppressive functions may be a prerequisite for initiation of prostate cancer.

On the other hand, the absence of distinctive basal cells in cancer and the many similarities in gene and protein expression between cancer cells and normal secretory epithelial cells have led some to suggest that cancer originates from secretory cells. Among the similarities between normal secretory and cancer cells is the expression of cytokeratins 8 and 18. However, in primary culture, cells from adenocarcinomas also

express cytokeratins 5 and 14 in addition to cytokeratins 8 and 18, like primary cultures from normal tissues, despite the fact that adenocarcinomas themselves do not express these basal cell cytokeratins. The explanation for this unexpected finding may relate to the plasticity of cytokeratin expression. Examples abound of modulation of cytokeratin expression either *in vitro* or *in vivo*. For instance, prostatic cells cultured from normal tissues express cytokeratins that are not expressed in prostatic tissues themselves (Sherwood *et al.* 1989). A particularly illuminating example of plasticity of cytokeratin expression is shown by the behavior of the SV40-immortalized human prostatic cell line, BPH-1. In culture, these cells express only the secretory cell cytokeratins 8 and 18. Yet when combined with urogenital sinus mesenchyme and grown under the kidney capsule of mice, BPH-1 cells form tumors after hormonal stimulation and express the basal cell cytokeratin 5 (Hayward *et al.* 2001). It is also noteworthy that even though tumor-derived primary cultures grown on collagen gels had different behavior compared with normal cells, they still expressed basal cytokeratins (Hall *et al.* 2002).

In fact, while basal cells *per se* are not present in prostatic adenocarcinomas, certain basal markers, such as CD44, are expressed in some prostate cancers (Liu *et al.* 2002). Furthermore, an analysis of the expression patterns of 119 cell surface markers (CD antigens) that were previously localized to basal or secretory cells of the normal prostatic epithelium revealed that prostate cancer cell lines expressed more basal-specific than secretory cell-specific molecules (Liu 2000). In addition, van Bokhoven *et al.* (2003) reported that 7 of 21 established prostate cancer cell lines expressed basal cell cytokeratin 5. All of the cell lines also expressed cytokeratins 8 and 18. Overall, it appears that attempting to categorize prostate cancer cells as basal or secretory is too simplistic, and in reality a pluripotent phenotype is probably a more accurate description.

Most investigators report that primary cell cultures derived from malignant tissues, like those from normal tissues, do not express AR or PSA (for review see Peehl 2004). While this may be appropriate for normal cultures if they are more like basal cells than fully differentiated secretory cells, this is not typical of cancer cells, which express both AR and PSA in adenocarcinomas (albeit heterogeneously). One interpretation may be that, as for normal cells, standard culture conditions are insufficient to maintain expression of differentiated functions such as expression of AR and PSA. Many established prostate cancer cell lines also do not express AR or PSA; of those that do,

such as LNCaP, LAPC-4 and MDA PCa 2, AR is commonly mutated (van Bokhoven *et al.* 2003).

ERs are also expressed by normal and malignant prostatic cells. ER α was expressed by all normal and cancer-derived prostatic epithelial primary cultures analyzed by Pasquali *et al.* (2001). However, several of the cancer-derived cultures expressed ER α variants, as has been found in established prostate cancer cell lines (Lau *et al.* 2000). ER β , expressed by basal epithelial cells in tissues (Leav *et al.* 2001), was expressed in primary cultures derived from normal tissues (Lau *et al.* 2000, Pasquali *et al.* 2001). ER β is for the most part absent in prostate cancer tissues, and ER β was expressed in only one of six primary cultures of prostate cancer cells (Pasquali *et al.* 2001). The ER β gene promoter is hypermethylated in DU 145 and LNCaP prostate cancer cell lines, preventing expression (Nojima *et al.* 2001); whether methylation is responsible for lack of expression of ER β by primary cultures is unknown. The biological significance of loss of ER β from prostatic cancer cells is suggested by the phenotype of ER β knock-out mice (Krege *et al.* 1998). These mice exhibit hyperplasia with aging, suggesting absence of anti-proliferative effects of estrogen or other ligands of ER β . The role of ER β in prostate cancer has been discussed by Signoretti & Loda (2001).

Cytogenetics

Chromosomal aberrations in prostate cancer are numerous and complex (Brothman *et al.* 1999, Paris *et al.* 2003). This is generally true of established prostate cancer cell lines as well, although karyotypes in the near-diploid, -triploid or -tetraploid are characteristic of a few cell lines (van Bokhoven *et al.* 2003). In contrast, chromosomal abnormalities in primary cultures derived from prostatic adenocarcinomas have been difficult to detect (for review see Peehl 2004). Primary cultures derived from adenocarcinomas are often diploid by standard karyotypic analyses. Some have interpreted this finding as suggesting that the so-called cancer cultures are in fact comprised of normal cells, either because of misdiagnosis of the tissue of origin or due to outgrowth of normal instead of malignant cells. Indeed, even with the most carefully characterized tissues, it is not possible to rule out the presence of some normal glands within the adenocarcinoma of origin. However, other types of genetic analyses show that cancer-derived cells do in fact have chromosomal changes, albeit at lower frequencies than present in the tumors of origin. As has been suggested, it is quite possible that current culture conditions do

not support the survival of the most genetically aberrant cancer cells *in vitro*.

Proliferation of cancer cells

In culture, cancer-derived primary cultures of prostatic epithelial cells generally respond similarly to normal cells to growth regulatory molecules, although individual cancer cell cultures that grow independently of EGF or hydrocortisone, or are resistant to growth-inhibitory factors such as vitamin D, have been reported (Peehl *et al.* 1989, Chopra *et al.* 1996, Gommersall *et al.* 2004). While dysregulation of signaling pathways involved in growth control is widely observed in prostate cancer cell lines, much less evidence of such dysregulation is seen in primary cultures of cancer cells. This could reflect fewer studies with primary cancer cultures, the inability of primary cultures to reflect the *in vivo* situation, selection of dysregulation by long-term culture of cell lines, or the fact that most primary cultures are derived from primary adenocarcinomas instead of metastases like the cell lines. Many of the dysregulated proliferation pathways implicated in cancer involve receptor kinases and, as pointed out in a recent review, studies of kinase activation in primary cultures of prostatic epithelial cultures are remarkably few (Maroni *et al.* 2004). Similarly, studies of phosphatase activity in primary cultures are lacking, although one group reported that the kinase-associated phosphatase KAP/Cdi1 was expressed at low levels in normal prostate tissues and cell cultures, and at high levels in prostate cancer and prostate cancer cell lines (Lee *et al.* 2000). Repression of KAP/Cdi1 in cancer cells reduced the number of cells in S-phase and reduced tumorigenic potential.

Normal primary cultures were used to demonstrate that overexpression of the co-chaperone protein Cdc37, that targets and activates multiple kinases, drives proliferation, whereas loss of Cdc37 function arrests growth and leads to apoptosis (Schwarze *et al.* 2003). This observation is relevant to the development and treatment of prostate cancer, which has elevated levels of Cdc37 compared with normal prostate tissue (Stepanova *et al.* 2000). In a similar vein, other investigators showed that levels of cyclin D1, which forms a complex with and activates cyclin-dependent kinases to drive the progression through the cell cycle, were low in normal prostatic epithelial cultures compared with prostate cancer cell lines (Han *et al.* 1998). Perturbations in the cyclins and associated kinases may play a role in dysregulation of growth in cancer.

One growth factor whose levels are significantly increased in prostate cancer compared with normal

prostatic tissue is IL-6 (Hobisch *et al.* 2000, Giri *et al.* 2001), and IL-6 may be elevated in the serum of prostate cancer patients (Michalaki *et al.* 2004). Giri *et al.* (2001) found that IL-6 was secreted by normal prostatic cell cultures, at quantities similar to those secreted by some prostate cancer cell lines. Treatment with exogenous IL-6 induced phosphorylation and translocation of Stat-3 into the nucleus. Physiologically relevant concentrations of IL-6 stimulated growth. IL-6 receptors in prostate tissues have been localized by immunohistochemistry mainly to basal epithelial cells in normal tissues, with some staining in secretory epithelial cells and stromal cells as well (Giri *et al.* 2001). IL-6 receptors in cancer are expressed heterogeneously. Altogether, these findings support the concept that IL-6 acts as an autocrine growth factor in the prostatic epithelium, with increased activity in cancer.

FGF-6 is another mitogenic factor with increased expression in prostate cancer. Expression is low both in normal epithelia in prostatic tissues and in cultured normal cells compared with cancer tissues and cancer cell lines (Ropiquet *et al.* 2000). Both normal and malignant cells, however, express the receptor for FGF-6 (FGFR-4), and FGF-6 is a potent mitogen for both normal and malignant cells (Ropiquet *et al.* 2000).

IGF-binding protein (IGFBP)-2 is a major IGFBP in the prostate whose levels are increased in prostate cancer (Cohen *et al.* 1993). IGFBP-2 suppresses growth of many types of cells, including normal prostatic epithelial cells, but a novel stimulatory effect of IGFBP-2 on prostate cancer cell lines was recently shown (Moore *et al.* 2003). These findings suggest that elevated expression of IGFBP-2 may have a causal role in progression of prostate cancer.

The human prostate is innervated by the autonomic nervous system and receptors for neurotransmitters, such as muscarinic acetylcholine receptors, have been localized in the prostate. Some investigators have suggested a growth regulatory role for neurotransmitters. Rayford *et al.* (1997) compared the response to carbachol, an analog of acetylcholine, of primary cultures derived from cancer and non-malignant prostatic tissues and observed a dramatically higher response by the cancer cells. The proliferative effect of carbachol suggests that muscarinic receptors may play a role in prostate cancer growth.

Senescence of cancer cells

Like cells cultured from normal prostatic tissues, cells from primary adenocarcinomas of the prostate have a

finite lifespan *in vitro* (for review see Peehl 2004). The development of spontaneously established cell lines from primary adenocarcinomas is extremely rare. In fact, van Bokhoven *et al.* (2003) have shown that almost all of the few such reported lines are in fact derivatives of the common cancer cell lines. Telomerase was reportedly present in primary cultures from cancer (Vicentini *et al.* 2002) but, as in normal prostatic epithelial cell cultures, expression of telomerase is not associated with an immortal lifespan.

Adhesion, invasion and migration of cancer cells

The expression of many proteases associated with invasion and migration is increased in prostate cancer, and primary cultures have been used to investigate the biological effects of specific proteases. Among the proteases that have been most studied in primary cultures are matrix metalloproteinases (MMPs). Varani *et al.* (2001) placed normal prostatic epithelial and stromal cells into culture for 2 days, then evaluated enzymatic activity of MMPs in the conditioned media. They found that stromal cells secreted MMP-2, whereas epithelial cells secreted both MMP-2 and MMP-9. This is somewhat similar to others' findings with serially passaged primary cultures (Wilson *et al.* 2002), except that basal levels of MMP-2 and MMP-9 were generally undetected in both normal and malignant epithelial cells and only appeared after treatment with TGF β . Another analysis of primary cultures of normal and malignant cells showed expression of MMP-2, MT1-MMP, MT3-MMP and the tissue inhibitors of MMPs (TIMP)-1 and -2 (Zhang *et al.* 2002). MMP-7 also was expressed at significant levels in the primary cultures in this study.

Stearns *et al.* (2003c) found that IL-10 blocked MMP-2 and MT1-MMP transcription and protein synthesis in primary cultures from cancer, and IL-10 also suppressed IGF-I induction of MMP-2 and MT1-MMP. IL-10 inhibited the production of MMP-9 by controlling expression of TIMP-1. Primary cultures of cancer cells were used to show that this activity is the result of IL-10 signaling through tyrosine phosphorylation of the IL-10R α chain (Stearns *et al.* 2003a). The enhancement of IL-10 activity by proteasome inhibitors (Stearns *et al.* 2003b) suggests potential therapeutic activity of IL-10 in combination with proteasome inhibitors that are currently in clinical trials (Adams *et al.* 2000).

Primary cultures of epithelial cells from cancer grown in co-culture with bone marrow stromal cells

made MMP-1 and MMP-7 (Hart *et al.* 2002). Blocking enzymatic activity of these MMPs with inhibitors reduced the size of colonies formed by the epithelial cells. Other investigators found MMP-7 activity in prostate cancer cell lines, but did not detect expression of MMP-7 in normal primary cultures (Klein *et al.* 1999, Udayakumar *et al.* 2001). Expression of MMP-7 in cancer cells appeared to be induced by FGF-1, because when normal cells were transfected with the appropriate receptor and treated with FGF-1, MMP-7 was induced. These investigators concluded that aberrant expression of FGFR-1 in prostate cancer cells mediates induction of MMP-7 expression by FGF-1. It was previously reported by Lang *et al.* (1998) that both cancer and non-malignant cells invaded bone marrow stromal cells in co-culture similarly, so invasion is not specific to cancer cells.

Hall *et al.* (2002) found differences in the behavior of normal vs cancer-derived primary cultures in co-cultures with stromal cells in type I collagen gels that presumably reflect differential expression of proteases. Stromal cells were seeded in the collagen matrix, then epithelial cells were inoculated on the top of the collagen gel. If the stromal cells were from normal tissue, the normal epithelial cells formed tightly coherent colonies on top of the gel, then migrated into the gel. If the stromal cells were from adenocarcinomas, the normal epithelial cells formed a loosely associated layer of cells on the gel surface. Cancer-derived epithelial cells developed an elongated morphology and invaded into gels containing normal stromal cells. On gels with tumor-derived stromal cells, the cancer-derived epithelial cells grew as a monolayer on the gel surface. Contraction of the collagen gels also depended on the particular mix of normal and cancer-derived stromal and epithelial cells. E-cadherin expression was also strikingly different between normal and cancer-derived epithelial cells. It will be of interest to identify the molecular basis of these intriguing phenomena.

Plasminogen activators are reportedly upregulated in prostate cancer. Epithelial cells grown from cancer tissues made urokinase plasminogen activator when co-cultured with bone stromal cells; inhibiting enzymatic activity reduced colony size (Hart *et al.* 2002). Another study showed that DNA methylation was the underlying molecular mechanism responsible for lack of expression of urokinase plasminogen activator in primary cultures of normal prostatic epithelial cells (Pakneshan *et al.* 2003).

The generation of eicosanoids by metabolism of arachidonic acid via cyclooxygenase (COX), LOX or other pathways has been implicated in prostate cancer.

Thromboxane synthase converts the COX product, prostaglandin H₂, into thromboxane A₂. While thromboxane synthase is expressed in some prostate cancer cell lines and tissues and promotes cell motility, normal cultures do not express this enzyme, in line with little or no expression in the epithelium of normal prostatic tissues (Nie *et al.* 2004).

Hevin, an anti-adhesive extracellular matrix protein that is expressed by cultured normal human prostatic epithelial cells and in the normal prostatic epithelium, is downregulated in metastatic prostate cancer cell lines (Nelson *et al.* 1998). Similarly, hevin is present in primary adenocarcinomas of the prostate, but not in metastases to the lymph nodes (Nelson *et al.* 1998). Loss of this molecule is considered to be relevant to the ability of metastatic cells to track through the endothelium. In contrast, normal primary cells weakly express MUC18, a cell adhesion molecule, whereas expression in prostate cancer cell lines is stronger (Wu *et al.* 2001).

Annexin II is involved in maintaining calcium homeostasis and regulating the cytoskeleton and cell motility. Several groups reported reduced or lost expression of annexin II in prostate cancer (Chetcuti *et al.* 2001, Smitherman *et al.* 2004). Similarly, abundant annexin II was demonstrated in normal cultures, with less or no expression in several prostate cancer cell lines (Liu *et al.* 2003). Normal cells did not migrate across Boyden chamber membranes, while the cell lines did. Restoration of annexin II to the cancer cells significantly inhibited migration, pointing towards a functional association between loss of annexin II and increased migratory activity of prostate cancer.

Integrins mediate interactions between cells and extracellular matrix proteins. As cell surface receptors, integrins have roles in cell migration, proliferation, and regulation of gene transcription. Alterations of integrins in cancer have been associated with tumor growth, invasion and metastasis. The $\alpha_v\beta_3$ integrin is expressed by primary cultures of prostate cancer cells but not by normal cells (Zheng *et al.* 1999). This integrin mediates adhesion to and migration on vitronectin, and stimulation can result in invasion through basement membrane. Because of the specificity of expression of $\alpha_v\beta_3$ by cancer cells, this integrin may provide a cancer-specific target for therapy. As a first step in this direction, Chatterjee *et al.* (2001) demonstrated the induction of apoptosis in $\alpha_v\beta_3$ -expressing prostate cancer cell lines but not in $\alpha_v\beta_3$ -negative lung cells by cyclic Arg-Gly-Asp peptides that interfere with the integrin/focal adhesion kinase-mediated signal transduction pathway.

Steroid hormone metabolism

Dihydrotestosterone (DHT) is the active form of androgen in the prostate that binds to the AR. The reduction of testosterone to DHT by 5 α -reductase is therefore a critical step in controlling androgen-mediated events in the prostate. The expression and activity of 5 α -reductase *in vitro* have mainly been studied in cells cultured from BPH rather than from normal tissues, or in immortalized or established cell lines. Nevertheless, these studies show that one or both isoforms of 5 α -reductase are expressed by cultured prostatic epithelial cells, whereas 5 α -reductase type 2 is believed to be the predominant enzyme in the human prostatic epithelium (Berthaut *et al.* 1996, Delos *et al.* 1998).

Aldo-keto reductases (AKRs) can convert sex hormones such as androgens, estrogens and progestins into their cognate inactive metabolites or vice versa. Local expression of AKRs in the prostate may control ligand availability at the pre-receptor level. AKR1C2 (type 3 3 α -hydroxysteroid dehydrogenase) is expressed in the prostate. In primary cultures, high levels of AKR1C2 were found in cancer but not normal cells (Rizner *et al.* 2003). This finding, together with metabolic studies in prostate cancer cell lines, suggests that in prostate cancer cells, AKR1C2 acts as a 3-ketosteroid reductase to eliminate DHT, and thereby reduce activation of the AR. While this seems contradictory with current ideas regarding promotion of prostate cancer progression by DHT, perhaps elimination of DHT from the local tissue milieu by over-expression of AKR1C2 contributes to progression towards androgen-independence.

17 β -Hydroxysteroid dehydrogenase (17 β -HSD) is another androgen-metabolizing enzyme of interest in the prostate. Type 10 17 β -HSD was shown to catalyze the oxidation of diol, an almost inactive androgen, to DHT (He *et al.* 2003). Furthermore, primary cultures of prostate cancer cells had higher levels of 17 β -HSD than normal cells. These investigators concluded that 17 β -HSD may play a significant part in non-classic androgen synthesis in the prostate, with higher expression of 17 β -HSD in cancer cells leading to higher intratumor levels of DHT.

Other androgen-metabolizing enzymes in the prostate include type 5 17 β -HSD and type 1 3 β -HSD. Expression of both was localized to the basal epithelia of prostate glands, and in one study, both enzymes were found to be expressed by cultured normal prostatic epithelial cells (El-Alfy *et al.* 1999). However, in another study, 3 β -HSD was not constitutively expressed in normal cell cultures but was induced by

IL-4 or IL-13 (Gingras & Simard 1999). The enzyme 3 β -HSD converts the inactive androgen, dehydroepiandrosterone, to androstenedione (4-dione), and type 5 17 β -HSD reduces 4-dione to testosterone. It appears that basal cells may be responsible for local production of testosterone, with conversion to DHT occurring in the AR-positive secretory cells by the action of 5 α -reductase. The fact that 17 β -HSD is more prominent than 5 α -reductase in primary cultures of normal prostatic epithelial cells (Delos *et al.* 1998) presumably reflects a more basal- than secretory-like phenotype of these cells.

As is evident from the previous discussion, steroid hormone metabolism in the prostate is indeed complex, but primary cultures are helping to sort out the many interacting pathways. With the growing recognition of intraprostatic production of active androgens from inactive adrenal precursors, the relevance of these pathways to failure of androgen-ablation therapy to cure prostate cancer is high.

Other metabolic pathways

The expression of 15-LOX-2, involved in arachidonic acid metabolism, in normal secretory cells of the prostatic epithelium was discussed in a previous section. A pair of donor-matched normal and cancer-derived primary cultures was used to study the biological functions of 15-LOX-2 (Tang *et al.* 2002). Expression was high in the normal cells, as it is in secretory epithelial cells in the prostate. Expression was reduced in the matched cancer culture, and virtually absent in established prostate cancer cell lines, reflecting the downregulation of expression that occurs in prostate cancer tissues (Jack *et al.* 2000). In the normal cells, 15-LOX-2 expression was inversely correlated with cell cycle progression, consistent with its expression in non-proliferative secretory cells in the prostate. To gain information about the biological functions of 15-LOX-2, the effect of its metabolic product, 15(S)-HETE, on cellular proliferation and survival was monitored. Cancer cells were more sensitive to growth inhibitory and apoptotic effects of 15(S)-HETE, and restoration of 15-LOX-2 expression to prostate cancer cell lines induced cell cycle arrest. Overall, these data suggest that 15-LOX-2 is a negative cell regulator in the normal prostatic epithelium whose loss is advantageous to prostate cancer development.

The cytochrome P450-dependent monooxygenases CYP1A1 and CYP1A2 are phase 1 metabolic enzymes that are relevant to carcinogenesis. CYP1A1 was found to be inducible in both normal and cancer-derived primary cultures, as is typical of cells of adult tissues

(Sterling & Cutroneo 2004). CYP1A2 expression was absent but inducible in cancer cells, and was constitutively expressed in normal cells. These primary cultures should therefore provide a good model system for investigation of the molecular mechanisms of prostate cancer development and progression from metabolic activation of dietary and environmental carcinogens.

Angiogenesis

The ability to stimulate new blood vessel formation (angiogenesis) is critical to cancer progression. Primary cultures have been used to investigate processes relevant to physiological and pathological angiogenesis. Thrombospondin-1 (TSP-1) is an anti-angiogenic factor that is secreted in substantial quantities by cultured normal human prostatic epithelial cells (Doll *et al.* 2001). However, pro-angiogenic factors, including vascular endothelial growth factor (VEGF) and FGF-2, are also secreted by normal cells (Campbell *et al.* 1999, Doll *et al.* 2001). In short-term (2 day) cultures, TSP-1 outweighed VEGF/FGF-2 production and resulted in net anti-angiogenic activity. In serially passaged cultures, the balance shifted and the secretions became angiogenic. Short-term cultures derived from cancers were also angiogenic, mainly due to increased VEGF and FGF-2 and decreased TSP-1. Other angiogenic factors that were expressed inconsistently by normal and/or cancer-derived primary cultures included IL-8, GRO α , HGF, and TGF β (Doll *et al.* 2001). IGF-I increased secretion of VEGF by normal cells, dependent on phosphatidylinositol 3-kinase and MEK1/2 signaling pathways (Burroughs *et al.* 2003). IGF-I also increased expression of hypoxia-inducible factor-1 (HIF-1), but studies with prostate cancer cell lines suggested that an additional signal that is not stimulated by IGF-I in normal cells is needed for HIF-1 to stimulate transcription from the VEGF hypoxia response element in cancer cells. Indeed, another study showed that HIF-1 transcriptional regulation was low in normal prostatic epithelial cell cultures and increased in prostate cancer cell lines in association with increasing metastatic ability (Salnikow *et al.* 2000). High inducibility of HIF-1-dependent genes may be part of the reason for survival of aggressive cancer cells in hypoxic conditions.

Hyaluronidase is an enzyme that degrades the glycosaminoglycan, hyaluronan, into small fragments. Hyaluronan and its degradation products have been found to have biological properties consistent with tumor-promoting abilities, including promotion of cell migration and angiogenesis. Both hyaluronidase and hyaluronan were found to be increased in prostate

cancer compared with normal tissues (Lokeshwar *et al.* 2001), and recently, HYAL1 type hyaluronidase was shown to be an independent prognostic marker (Posey *et al.* 2003). When primary cultures of epithelial cells were examined, cancer-derived cells were noted to secrete higher amounts of both the enzyme and the glycosaminoglycan than cells from normal tissues (Lokeshwar *et al.* 2001). Furthermore, the pattern of higher expression of these tumor markers in cancers of higher Gleason grades was mimicked in cell cultures derived from high vs low grades. Primary cultures may serve as a useful model to further investigate the role of hyaluronidase-generated fragments of hyaluronan in cancer progression. The role of hyaluronan in tumor growth was highlighted by the impairment of growth and vascularization of a prostate cancer xenograft model when synthesis of hyaluronan was blocked (Simpson *et al.* 2002). Similarly, overexpression of HYAL1 in an orthotopic model of prostate cancer resulted in significantly increased numbers of metastases (Patel *et al.* 2002).

In another study, six of six cancer-derived primary cultures were found to have increased expression of inducible nitric oxide synthase (iNOS) mRNA compared with the paired non-neoplastic primary cultures (Wang J *et al.* 2003). It has been shown that increased levels of nitric oxide, produced by iNOS, can contribute to angiogenesis, growth and metastasis, and immune suppression. In this same study, the investigators also showed that iNOS protein levels were significantly higher in immunostained prostate cancer tissue sections than in adjacent benign epithelia.

Gene expression

Gene expression profiling and proteomics are providing novel insights into cancer-related traits. Comparisons of genetic expression profiles of prostatic adenocarcinomas with either normal tissues or BPH have been published by a number of groups (for review see De Marzo *et al.* 2004). Genetic profiling of prostate cancer cell lines in comparison with prostate cancer tissues has been performed by several investigators. It is interesting to note that Welsh *et al.* (2001) found only a small number of genes with concordant expression in established prostate cancer cell lines and malignant prostate tissues, and Bull *et al.* (2001) found that cell lines overexpressed fewer genes than did cancer tissues in comparison with normal tissues. Nevertheless, Dhanasekaran *et al.* (2001) found that cell lines clustered with malignant rather than with normal or benign tissues in microarray analyses.

While it seems that no similar analyses of primary cultures of prostate cancer cells in comparison with tissues have yet been reported, the genetic expression profile of cultured normal prostatic cells was compared with profiles of five xenograft-derived prostate cancer cell lines. A consensus class of genes that differed in all of the lines relative to the normal cells was identified (Glinsky *et al.* 2004). Many of these genes were similarly differentially expressed in prostate cancer tissues compared with normal tissues, indicating that these genes may indeed be relevant to prostate cancer.

One gene found to be overexpressed in prostate cancer compared with non-malignant tissues by cDNA microarray analysis was LIM kinase 1 (LIMK1) (Dhanasekaran *et al.* 2001). *In vitro* studies confirmed the lower expression of this kinase in normal prostatic epithelial cells compared with many prostate cancer cell lines (Davila *et al.* 2003). These studies also showed that reducing the expression of LIMK1, which is involved in reorganization of the actin cytoskeleton, abolished the invasiveness of cancer cells. A role for LIMK1 in mediating the invasive property of prostate cancer is therefore implicated, and LIMK1 may be a new therapeutic target.

Aberrant DNA methylation patterns are posited to be some of the earliest genomic changes in prostate cancer, and indeed, the frequency of hypermethylation of CpG islands was high in prostate cancer cell lines compared with normal cells (Yegnasubramanian *et al.* 2004). Hypermethylation of a select set of genes was shown to distinguish benign prostatic tissues from primary adenocarcinomas in this study.

Das *et al.* (2001) compared the expression pattern of fatty acid-binding proteins (FABPs) in cultured normal prostatic epithelial cells and prostate cancer cell lines. Interestingly, the elevation of L-FABP and I-FABP, and the downregulation of A-FABP and E-FABP, that were seen in the cancer cell lines compared with the normal cells were also noted in normal vs malignant prostatic tissues. Furthermore, B-FABP was elevated in only one of the cancer cell lines, LNCaP, considered to be more well-differentiated than other prostate cancer cell lines, and B-FABP was distinctively elevated only in well-differentiated prostate cancers compared with normal epithelium or poorly differentiated cancers. These results suggest that the normal primary cultures and cancer cell lines faithfully replicate the *in vivo* situation and will be useful models for further investigation of the role of FABPs in prostatic carcinogenesis.

Another gene, EBAG9/RCAS1, was found to be highly expressed in prostate cancer cell lines compared with normal cells (Takahashi *et al.* 2003). The protein

product of this gene is a cancer cell surface antigen implicated in immune escape. Besides the implications of overexpression of this gene in cancer progression, localization of the protein on the cell surface makes EBAG9/RCAS1 a potential target for antibody-mediated therapy or for selection of cancer cells for experimental purposes.

Metastatic prostate cancer cells

No method has been devised for reproducible primary culture of prostatic cancer cells from bony or soft tissue metastases. The small number of immortal metastatic cancer cell lines that exist represent rare successes from hundreds of attempts to establish such lines. There are a few reports of short-term cultures in which epithelial cells were isolated from bone marrow aspirates and expanded somewhat in culture (Pantel *et al.* 1995). One group purportedly established prostatic primary cultures from a number of metastatic sites and investigated expression of β -2 microglobulin as a marker of metastatic disease, but little information was provided about the culture methodology or characterization of the cultures (Abdul & Hoosein 2000). However, in a recent study of the proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors, 46 patients with prostate cancer were included (Solakoglu *et al.* 2002). Bone marrow cells were plated on extracellular matrix and the number of cytokeratin-positive (epithelial) cells was determined at each passage. Cytokeratin-positive cells were seen in cytospin preparations of the bone marrow aspirates in 24% of the prostate cancer patients; after culture, this percentage rose to 94% of the attached cell population. In fact, the highest median number of *in vitro*-expanded, cytokeratin-positive cells was obtained from prostate cancer patients in comparison with individuals with other types of tumors. In a previous study using similar methodology but including transfection with the SV40 T-Ag to create immortal cell lines, it is interesting to note that cells not expressing the T-Ag did not survive after crisis (Putz *et al.* 1999). This phenomenon emphasizes the point that immortality is not necessarily an intrinsic feature even of metastatic cells. Further development of the methodology described in these publications may lead to enhanced ability to capture and study metastatic prostatic epithelial cells *in vitro*.

Preclinical studies with primary cultures

While the cell in which cancer originates in the normal epithelium (stem, basal, transit amplifying or secretory)

remains unknown, and primary cultures may include a variable mixture of these diverse phenotypes, primary cultures derived from normal tissues have been used to study numerous aspects of normal prostatic biology relevant to carcinogenesis and chemoprevention. Primary cultures of normal prostatic epithelial cells are often compared with established prostate cancer cell lines to generate evidence of cancer-specific traits. When normal cultures are compared with established cell lines, many differences indeed are seen. However, it is difficult to know whether observed differences really represent differences between normal and malignant cells, or simply reflect the different culture conditions in which the primary cultures vs the cell lines are generally maintained. The most informative studies are those in which donor-matched neoplastic and non-neoplastic primary cultures of prostate cells are compared, because these are grown under identical conditions and inter-individual variation among donors is controlled. Barring the availability of donor-matched pairs, primary cultures of normal and cancer cells from different individuals are still valuable because at least they are grown in identical culture conditions. Features that have been reported to differ between primary cultures derived from adenocarcinomas vs normal tissues are listed in Table 5. Another advantage of primary cultures is that the generality of a given observation can be confirmed by studying cell cultures derived from numerous individuals, and cancer cultures can be derived from adenocarcinomas representing a range of aggressiveness. In the following sections, preclinical studies with primary cultures that are relevant to clinical application are discussed.

Chemoprevention

Ideally, a chemopreventive agent might kill cancer cells as they arise while having minimal or no effect on normal cells. Gupta *et al.* (2001) used normal primary cultures and established cancer cell lines to show that apigenin, a flavonoid that is abundant in fruits and vegetables, may exhibit this type of differential activity. While apigenin had moderate growth-inhibitory activity on normal cells, cancer cells underwent apoptosis when treated with apigenin. Similarly, the Bowman-Birk inhibitor, a soybean-derived serine protease inhibitor with demonstrated anti-cancer activity both *in vitro* and *in vivo*, was found to inhibit the growth of several prostate cancer cell lines but not normal prostatic epithelial cells (Kennedy & Wan 2002). Allyl isothiocyanate, a constituent of cruciferous vegetables, caused G₂/M arrest and induced

apoptosis in several prostate cancer cell lines, while minimally affecting the survival of normal prostatic epithelial cells (Xiao *et al.* 2003).

Tomato, soy and tea polyphenols (genistein, quercetin, biochanin A, daidzein and others) have been proposed as chemopreventive agents. Polyphenols inhibited the growth of normal prostatic epithelial cells (Hedlund *et al.* 2003), with inhibition of the IGF-I signal transduction pathway implicated in this activity (Klein & Fischer 2002, Wang S *et al.* 2003). These findings provide further support for potential chemopreventive activity of polyphenols because IGF-I activity has been linked to increased prostate cancer risk (Pollak *et al.* 2004). In many cases, the growth inhibitory concentrations of these compounds were within the range that can be attained through dietary consumption (Hedlund *et al.* 2003). In contrast, prostate cancer cell lines show variable responses to polyphenols, and in many cases require supraphysiological concentrations for maximal effect (Hedlund *et al.* 2003).

The effects of vitamin D compounds on prostate cells are also consistent with chemopreventive activity (for review see Krishnan *et al.* 2003). While vitamin D does not exhibit preferential activity on prostate cancer vs normal cells, vitamin D inhibits growth and increases differentiation of primary cultures derived from normal and malignant tissues and cancer cell lines. Epidemiological data link low levels of vitamin D to increased risk of prostate cancer. An important discovery was the finding that normal prostatic epithelial cells themselves express the enzyme, vitamin D 1 α -hydroxylase (CYP27B1), that synthesizes the active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃. This finding implies that local production of 1,25-dihydroxyvitamin D₃ is biologically significant. Furthermore, the finding that primary cultures of prostate cancer cells and prostate cancer cell lines are deficient in CYP27B1 suggests that elimination of local production of vitamin D may play a role in prostatic carcinogenesis. Chemopreventive strategies might be focused on restoring or compensating for loss of local synthesis of active vitamin D in the premalignant or early stages of prostatic cancer development.

Retinoids also modulate growth and differentiation of prostatic epithelial cells and various retinoids have been tested for chemopreventive and/or chemotherapeutic activity against prostate cancer (for review see Peehl & Feldman 2003). Primary cultures have been used to study retinoid metabolism, with some thought-provoking findings. Lecithin:retinol acyltransferase (LRAT) is an enzyme that metabolizes retinol to retinyl esters, the primary intracellular storage form of

Table 5 Features that distinguish primary cultures of epithelial cells derived from adenocarcinomas vs normal tissues

Feature	Reference
ER α variants	Lau <i>et al.</i> (2000)
ER β	Pasquali <i>et al.</i> (2001)
Cytogenetics	Reviewed in Peehl (2004)
Growth factor independence	Peehl <i>et al.</i> (1989), Chopra <i>et al.</i> (1996)
Resistance to growth inhibitory factors	Gommersall <i>et al.</i> (2004)
Response to acetyl choline analog	Rayford <i>et al.</i> (1997)
Behavior in collagen gels	Hall <i>et al.</i> (2002)
$\alpha_v\beta_3$ integrin	Zheng <i>et al.</i> (1999)
AKR1C2	Rizner <i>et al.</i> (2003)
Type 10 17 β -HSD	He <i>et al.</i> (2003)
15-LOX-2	Tang <i>et al.</i> (2002)
CYP1A2	Sterling & Cutroneo (2004)
HYAL1	Lokeshwar <i>et al.</i> (2001)
iNOS	Wang J <i>et al.</i> (2003)
LRAT	Guo <i>et al.</i> (2002)
Vitamin D 1 α (OH)ase	Hsu <i>et al.</i> (2000)
SSTR1/SSTR2	Sinisi <i>et al.</i> (1997)
NMR spectra	Yacoe <i>et al.</i> (1991)
Hyaluronan	Lokeshwar <i>et al.</i> (2001)

vitamin A. Retinol esterification occurred normally in primary cultures of prostatic epithelial cells from normal tissues (Lewis & Hochadel 1999, Guo *et al.* 2002). Correspondingly, LRAT mRNA transcripts were present in these cells. In comparison, LRAT mRNA was considerably reduced in both primary cultures derived from prostatic adenocarcinomas and in prostate cancer cell lines and conversion of retinol to retinyl esters was markedly reduced (Guo *et al.* 2002). These findings imply that prostate cancer cells are retinoid-deficient relative to normal cells, implicating aberrant retinoid metabolism in the process of prostatic carcinogenesis. It is noteworthy that two enzymes, CYP27B1 and LRAT, involved in creating active metabolites of the potent growth-suppressing and differentiation-promoting vitamins A and D, are aberrant in prostate cancer cells, both in primary culture and in established cell lines. Furthermore, these deficiencies were noted even in primary cultures derived from well-differentiated cancers (Gleason grade 3), implying that these are early steps in prostate cancer development. As suggested in reference to loss of the ability of prostate cancer cells to synthesize the active metabolite of vitamin D, strategies to restore or compensate for loss of LRAT activity may be a new chemopreventive mechanism.

Somewhat surprisingly, antagonists of retinoic acid receptors were reportedly potent growth inhibitors of

primary cultures of prostate cancer cells as well as of cancer cell lines (Hammond *et al.* 2001). While no mechanistic explanation for this unexpected finding was presented, it is possible that this result relates to the apparent growth stimulatory effect of low concentrations of retinoic acid that have been observed in primary cultures of prostatic epithelial cells (Peehl *et al.* 1993).

Consumption of lycopene, a red carotenoid obtained primarily from tomatoes, has been inversely correlated with risk of prostate cancer (Etminan *et al.* 2004). In studies with primary cultures of normal prostatic epithelial cells, lycopene was found to inhibit proliferation and block cell cycle progression at physiologically relevant concentrations (Obermuller-Jevic *et al.* 2003).

Primary cultures have also been used to explore potential interactions between putative chemopreventive agents. Synergistic growth inhibition of normal prostate cells by vitamin D and genistein was shown (Rao *et al.* 2002), raising the possibility of dietary supplementation with vitamin D and genistein as an effective prostate cancer prevention strategy. Retinoic acid and vitamin D also synergistically inhibit the growth of primary cultures of prostatic epithelial cells, both normal and malignant (Peehl *et al.* 1995).

Some epidemiological studies have also linked consumption of non-steroidal anti-inflammatory drugs (NSAIDs) with lower risk of prostate cancer (Mahmud *et al.* 2004). The basis of this effect is believed to be inhibition of COX-2, which synthesizes prostaglandins that stimulate the growth of prostate cancer. The pattern of expression of COX-2 in human prostate tissues is variable in different reports, as is expression in cultured cells. Some groups reported that cultured normal prostatic epithelial cells express high levels of this enzyme (Lim *et al.* 1999, Subbarayan *et al.* 2001), whereas others found low expression (Hsu *et al.* 2000). NSAIDs may exert chemopreventive activity by lowering the production of mitogenic prostaglandins in the prostatic epithelium.

In a previous section, the attenuated activation of the tumor suppressor p53 in normal prostatic epithelial cells in response to DNA damage was discussed. Given the anti-cancer functions of p53, restoration of p53 activity might be a viable chemoprevention strategy. That this may be feasible was demonstrated by the upregulation and activation of p53 in normal prostatic epithelial cells by leptomycin B, an antifungal agent that inhibits protein export from the nucleus to the cytoplasm and thus prevents the proteasomal degradation of p53 (Lecane *et al.* 2003). While leptomycin B is too toxic for clinical use, new proteasomal inhibitors

that are in clinical trials to treat cancer (Adams *et al.* 2000) may achieve the same effect on p53 and find a use in chemoprevention as well as chemotherapy. Another compound that causes significant upregulation of p53 in primary cultures of either normal or malignant prostatic epithelial cells is triptolide (Kiviharju *et al.* 2002). A natural product in Phase I clinical trials, triptolide at low concentrations induces senescence and, at high concentrations, causes apoptosis of prostatic epithelial cells. These dose-dependent effects might be taken advantage of in designing chemopreventive vs chemotherapeutic strategies with this compound.

Chemotherapy

Screening experimental chemotherapeutic drugs has largely been accomplished with prostate cancer cell lines, although in the early 1990s a large National Cancer Institute-sponsored screen of synthetic and natural products was carried out with primary cultures of prostate cancer cells derived from adenocarcinomas of differing Gleason histopathological patterns (Peehl *et al.* 1994a). It is felt that the rapid proliferative rate of many of the prostate cancer cell lines leads to inappropriate selection of drugs with anti-proliferative activity, which might not exhibit much efficacy on generally slowly growing prostatic adenocarcinomas. Primary cultures may prove to be a more realistic model.

Apoptosis is a critical factor balancing proliferation in normal epithelium and maintaining tissue homeostasis. The rate of apoptosis is decreased in malignant epithelium (Berges *et al.* 1995). Restoring apoptosis is a therapeutic strategy, and requires an understanding of mechanisms of apoptosis in prostatic epithelial cells (Isaacs 2000). Thapsigargin, a sesquiterpene lactone that selectively inhibits the sarcoplasmic reticulum/endoplasmic reticulum calcium-dependent ATPase, induced apoptosis in primary cultures of cancer cells (Lin *et al.* 1997). An important feature of this study was that the cancer cells were made quiescent by maintaining at confluency in thymidine-free medium. This state more closely mimics that of cancer *in vivo*, which typically has a low proliferative index, than that of cells in standard culture media that are rapidly proliferating. The low proliferation rate of most prostate cancers requires proliferation-independent cytotoxic therapies such as provided by thapsigargin.

Coffey *et al.* (2001) used primary cultures of normal and cancer-derived cells to investigate molecular mechanisms of apoptosis. Their study was based on their preliminary findings that procaspase-3 levels were

decreased in prostate cancer tissues. Since activation of caspases is often essential for apoptosis, lack of caspases could have a detrimental effect on efficacy of apoptosis-inducing therapies. Coffey *et al.* found that pretreatment with diethyl-maleate (DEM) prior to exposure to Fas antibody, etoposide or radiation increased sensitivity to induction of apoptosis by these agents. This effect was attributed to DEM's depletion of cytoplasmic levels of glutathione, which protects cells from oxidative damage. Increased procaspase-3 and caspase-8 were also associated with DEM treatment. Such studies are relevant to the development of strategies to sensitize prostate cancer to chemical or radiation therapy.

Recent findings suggest that quinazoline-based α_1 -adrenoceptor antagonists, already in clinical use for the treatment of BPH, may have potential for therapy of prostate cancer as well. These antagonists induce apoptosis in prostate cancer cell lines via an unidentified but α_1 -adrenoceptor-independent mechanism (Benning & Kyprianou 2002). Of significance is the observation that normal prostatic epithelial cells, in comparison with the cancer cell lines, are very resistant to the apoptotic effects of the antagonists (Benning & Kyprianou 2002). The apparent selective activity of these drugs on cancer cells is clearly a desirable feature. Thebault *et al.* (2003) showed that α_1 -adrenergic receptor-stimulated signaling in normal prostate cells is preferentially coupled to store-independent transient receptor potential (TRP) channels, as opposed to store-operated TRP channels in the prostate cancer cell line LNCaP. It should be noted, however, that other investigators found no evidence for functional α_1 -adrenergic receptors in cultured normal prostatic epithelial cells, in line with the apparent absence of these receptors in epithelia of prostatic tissues (Kanagawa *et al.* 2003, Marinese *et al.* 2003). Nevertheless, these investigators suggest that isoform-specific inhibitors of TRP channels may be useful for treating prostate cancer.

In another study, treatment with neutral endopeptidase (NEP) was found to increase protein kinase C- δ in PC-3 but not normal prostate cells, and to preferentially sensitize PC-3 cells to etoposide-induced apoptosis, implying that NEP might augment chemosensitivity in prostate cancer with minimal toxicity in normal tissues (Sumitomo *et al.* 2004).

Selenium has been primarily investigated for chemoprevention of prostate cancer, but new data suggest that selenium may also be useful for treatment. Ghosh (2004) demonstrated that normal prostatic epithelial cells were markedly resistant to the apoptotic-inducing properties of sodium selenite, whereas prostate cancer

cell lines were very sensitive. This differential sensitivity between normal and cancer cells to selenite was hypothesized to be linked to different metabolism of arachidonic acid.

Like selenium, vitamin D compounds are being considered both to prevent (discussed above) and treat prostate cancer (for review see Krishnan *et al.* 2003). Hypercalcemia caused by treatment with the active metabolite, 1,25-dihydroxyvitamin D₃, is being circumvented in a variety of ways, including novel dosing regimes, use of analogs, and combination therapy with other drugs. Results from studies with primary cultures of cancer cells suggest that combination therapy of 1,25-dihydroxyvitamin D₃ with ketoconazole, an inhibitor of P450 enzymes used for second-line androgen-ablation therapy, may be a useful clinical strategy (Peehl *et al.* 2002). Ketoconazole simultaneously blocks androgen production while inhibiting metabolism of 1,25-dihydroxyvitamin D₃, and has other growth-inhibitory effects on prostate cancer cells independently of these two activities.

The EGF receptor tyrosine kinase inhibitor ZD1839 has shown encouraging activity in clinical trials against a variety of cancers. Primary cultures derived from prostate cancers were used to demonstrate that EGF receptors were present and phosphorylated, and that ZD1839 reduced receptor auto- and EGF-induced phosphorylation in conjunction with inhibition of cell growth (Vicentini *et al.* 2003). These results suggest that ZD1839 may have potential for treating prostate cancer.

Based on the observation that p53 is non-functional in primary cultures of prostate epithelial cells (discussed in a previous section) and often mutated in established prostate cancer cell lines (van Bokhoven *et al.* 2003) and prostatic adenocarcinomas (Downing *et al.* 2001), drugs that induce apoptosis by p53-independent mechanisms might be more active than those that require p53 activity. Brefeldin A is one such compound, and it is a potent inducer of apoptosis in primary cultures of cancer cells (Wallen *et al.* 2000).

Analogues of somatostatin have been tested in clinical trials against prostate cancer, with limited efficacy. Results from studies by Sinisi *et al.* (1997) with primary cultures provide a possible explanation for these less than promising clinical results. These investigators characterized the somatostatin receptor subtypes in primary cultures from normal and malignant prostatic tissues, and observed that the subtype SSTR1 was expressed only in cancer cells while SSTR2 was found only in normal cells. The use of SSTR2-selective analogs in the clinical trials, therefore, presumably

targeted the wrong cell population, and better efficacy might be achieved with SSTR1-selective analogs.

Ideas for new therapeutic strategies are taking advantage of novel information derived from genetic profiling of normal vs malignant tissues. One gene found overexpressed in prostate cancer, particularly androgen-independent cancer, was fatty acid synthase (FAS). This enzyme converts dietary carbohydrate or protein to fat. The resistance of normal prostatic cells to the growth-inhibitory and apoptosis-inducing effects of the FAS inhibitor, cerulenin, compared with prostate cancer cell lines suggests that FAS may serve as a target for antimetabolite therapy in prostate cancer (Pizer *et al.* 2001).

Cytotoxin therapy

Cytotoxins composed of tumor-selective cytokines coupled to a toxin moiety are a new class of molecular targeting agents. On the basis of studies with primary cultures and established prostate cancer cell lines, Husain *et al.* (2003) proposed that administration of IL-4 fused with *Pseudomonas* exotoxin (IL4-CTx) may provide an effective therapy for prostate cancer. These investigators demonstrated that IL-4 receptors are present in prostate cancer tissues as well as on primary cultures and cell lines, and that all of the cultured cells were very sensitive to IL4-CTx cytotoxicity. IL4-CTx caused regression of xenografts of several of the prostate cancer cell lines, demonstrating pre-clinical efficacy of this approach.

Radiation therapy

Given that radiotherapy is a widely used treatment option for men with prostate cancer, it is surprising that relatively little has been done to investigate radiation sensitivity and response of primary cultures. Bromfield *et al.* (2003) measured clonogenic survival and apoptosis of primary cultures of irradiated normal prostatic epithelial cells, but the responses of the normal cells were compared with those of established prostate cancer cell lines rather than to primary cultures of cancer cells. However, normal cells were more sensitive than the cancer cell lines, suggesting radioresistance of the latter.

In another study, Dunlap *et al.* (2003) determined the sensitivity of primary cultures of cancer-derived cells to ionizing radiation, then showed that pre-treatment with vitamin D potentiated the growth-inhibitory effect of radiation. They used normal prostatic stromal cells to show that the normal cells were substantially more resistant to combined treatment than prostate cancer cells. Thus, vitamin D might

be a biological response modifier that could permit a reduction in the dose of radiation used to treat prostate cancer and reduce treatment-related morbidity.

Gene therapy

Primary cultures of prostate cancer cells were used to test the effects of a recombinant adenovirus vector expressing wild type p53 (Asgari *et al.* 1997). The primary cultures were growth-inhibited, as was the growth of a xenograft of the DU 145 prostate cancer cell line, showing preclinical activity of this gene therapy approach. Another group showed that complexing adenovirus-based plasmids to cationic liposomes led to high levels of gene transfer and expression in primary cultures of epithelial cells derived from prostatic adenocarcinomas (Vieweg *et al.* 1995).

The cytokine IL-24, when expressed by a replication-incompetent adenovirus (Ad.mda-7), shows broad specificity for apoptotic effects on cancer cells vs normal cells. Results of studies with normal prostatic epithelial cells and prostate cancer cell lines reiterated this general theme, with growth suppression and apoptosis induced by Ad.mda-7 evident only in the cancer cells, despite similar kinetics of infection and expression of IL-24 in both the normal and cancer cells (Lebedeva *et al.* 2003).

Imaging

Non-invasive imaging technologies are being developed to obtain reliable markers for diagnosis of prostate cancer. One of the technologies that shows promise is proton magnetic resonance spectroscopic imaging (¹H MRSI). ¹H MRSI spectra in prostate cancer tissues typically exhibit low levels of citrate and increased levels of total choline (consisting of phosphocholine, glycerophosphocholine and free choline). Similarly, ¹H MRSI spectra of normal prostatic epithelial cell cultures and prostate cancer cell lines revealed elevated levels of phosphocholine and glycerophosphocholine in the cancer cells (Ackerstaff *et al.* 2001). Effects of cell density, doubling time or other culture conditions as a factor in this differential phenotype between the primary cultures and cell lines were ruled out, suggesting that the differences are in fact attributable to alterations of phospholipid metabolism in cancer. An earlier investigation of ¹H NMR spectra in primary cultures of prostate cancer compared with normal cells also suggested that cancer cells had smaller amounts of citrate than normal cells (Yacoe *et al.* 1991). These *in vitro* studies support further investigation of the choline signal as an

imaging marker to detect malignant prostate cells for diagnosis and treatment.

Conclusions

The contribution of primary cultures to our knowledge of normal and malignant prostate biology has perhaps been under-appreciated. Challenges that remain in order for primary cultures to be fully utilized as *in vitro* models include the isolation, culture and characterization of stem cells, and development of methods that induce or maintain the fully differentiated secretory epithelial cell phenotype, include robust androgen responsiveness.

The many emerging cancer-related traits expressed by primary cultures derived from adenocarcinomas emphasize the value of this model system in the repertoire of tools for prostate cancer research. However, the changing nature of prostate cancer at diagnosis will have a significant impact on our ability to establish primary cultures from adenocarcinomas unless some new tools are developed. Twenty years ago, the mean volume of primary adenocarcinomas of the prostate diagnosed at Stanford was 5.3 cc (Stamey *et al.* 2004). Even given that relatively large tumor size, gross dissection of malignant tissues for culture was difficult, and in my personal experience, subsequent histopathological analysis showed that even our expert pathologist had accessed tissues composed of >90% cancer only about half of the time. Now, the mean volume of cancers at diagnosis is 2.4 cc. Correspondingly, our success rate at grossly dissecting tissues that are mostly cancer has significantly dropped. Unless a cell surface marker that can reliably sort live cancer from non-malignant cells is identified soon, primary cultures derived from prostatic adenocarcinomas will become a scarce commodity. I feel that this will be a great loss to the research community, considering the unique information that has been and could be gained from such cultures.

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