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The Impact of Cell Adhesion Changes on Proliferation and Survival During Prostate Cancer Development and Progression

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Abstract In the normal prostate epithelium, androgen receptor (AR) negative basal epithelial cells adhere to the substratum, while AR expressing secretory cells lose substratum adhesion. In contrast, prostate cancer cells both express AR and adhere to a tumor basement membrane. In this review, we describe the differential expression of integrins, growth factor receptors (GFRs), and AR in normal and cancerous epithelium. In addition, we discuss how signals from integrins, GFRs, and AR are integrated to regulate the proliferation and survival of normal and malignant prostate epithelial cells. While cell adhesion is likely of great importance when considering therapeutic approaches for treatment of metastatic prostate cancer, no data on integrin expression are available from tissues of prostate cancer metastasis. However, several drug targets that are upregulated after androgen ablative therapy regulate cell adhesion and thus novel targeted therapies indirectly interfere with cell adhesion mechanisms in prostate cancer cells. J. Cell. Biochem. 99: 345–361, 2006.
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Key words: androgen receptor; integrins; prostate cancer; growth factor receptors; signal transduction

INTERACTIONS BETWEEN SUBSTRATUM ADHESION, PARACRINE GROWTH FACTORS, AND ANDROGEN IN NORMAL PROSTATE EPITHELIUM

In the human and mouse adult prostate epithelium, p63 expressing basal cells differentiate into secretory cells, transitioning through an intermediate/transiently proliferating cellular compartment. During differentiation the cells change their cytokeratin expression. Basal cells are K14 and weakly K5 positive, intermediate cells are K5 and K18 positive, and secretory cells are K18 and K8 positive. Differentiation is also accompanied by the formation of a suprabasally located secretory cell layer, loss in adhesion to the substratum, and gain in expression of the androgen receptor (AR). Thus, in normal epithelium, there is an inverse relationship between cell adhesion and AR expression.

Basal Epithelial Compartment

During prostate development androgen-regulated stromal factors, named andromedins, interact with non-androgen-regulated growth factors to stimulate epithelial morphogenesis. In contrast to basal epithelial cells, stromal cells express AR and thus proliferation, survival, and branching morphogenesis of basal epithelial cells are indirectly regulated by androgens through the prostate stroma. The responsible stromal factors include FGF7, FGF10, IGF, and HGF [Thomson, 2001; Donjacour et al., 2003; Knudsen and Edlund, 2004]. In particular, forced expression of FGF7 in the prostate stroma of transgenic mice caused epithelial hyperplasia, [Foster et al., 2002]. A recent mouse model demonstrated that stromal growth factors are also regulated via an autocrine loop that involves TGF-β in the prostate stroma

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[Bhowmick et al., 2004]. Knockout of the TGF-β type II receptor resulted in increased HGF secretion by stromal cells and the development of intraductal carcinoma.

Integrins comprise a family of cell adhesion receptors that regulate the attachment of epithelial cells to the basement membrane, also called substratum. They function as co-receptors of GFRs, allowing effective transduction of signals from the basal cell surface to the cytoplasm and nucleus. Thus, integrins are involved through multiple distinct pathways and networks in the regulation of prostate epithelial growth and oncogenesis. Here we examine integrin-mediated pathways that are specific to normal prostate basal epithelial cells and to prostate cancer cells, since these are the only two cell types that adhere to the substratum.

In the normal human prostate gland, AR-deficient basal cells adhere to substratum containing collagen IV, collagen VII, laminin 5, and laminin 10/11 [Knox et al., 1994]. Adhesion to collagen IV is mediated by integrin α2β1, while adhesion to collagen VII and laminin 5 is mediated through α6β4 and α3β1. Studies in α3, α6, β4 or laminin 5 null mice suggest a high level of redundancy of these components for basal cell function [Ryan et al., 1999; DiPersio et al., 2000]. All of these mice develop a severe blistering phenotype in the skin and oral epithelium following birth, likely caused by abrasive action. However, the normal architecture of stratified epithelial differentiation in the skin is maintained prior to birth. Extensive apoptotic cell death, termed anoikis, occurs in blisters where epithelial cells detach from the substratum, clearly demonstrating the important role of substratum adhesion for cell survival of basal cells. In contrast to laminin 5 null mice, collagen IV deficient mice fail to stabilize basement membranes [Poschl et al., 2004]. Surprisingly, integrin-α2 null mice do not develop a blister phenotype [Chen et al., 2002]. In general, the prostates of viable integrin null mice at birth were not examined and since knockouts cause neonatal lethality, tissue transplantation is necessary to determine the role of integrins during branching morphogenesis and epithelial differentiation of the prostate.

Attachment of quiescent epithelial cells to the substratum occurs primarily via integrins α6β4 in hemidesmosomes. The crosstalk between hemidesmosomes, which anchor cells to the substratum and E-cadherin-based cell–cell interactions helps to limit proliferation. During cell division there is a temporary disruption of cell–cell and α6β4 hemidesmosomal interactions and this removes the brake that acts to suppress growth and migration of cells. When α6β4 interactions are disrupted, engagement of α3β1 integrins may increase temporarily to support cell proliferation. The α3β1 integrins are typically localized within the basal-lateral cell membrane and may not be fully engaged through substratum binding in non-proliferating basal cells [Yanez-Mo et al., 2001]. Thus, integrin utilization and not just integrin expression levels determine interactions of cells with the substratum and regulate cell proliferation and migration.

Intermediate/Transiently Proliferating Cell Compartment

Prostate basal cells are the first epithelial cell type in the prostate to appear during development and are responsible for ductal morphogenesis. We now appreciate that basal cells differentiate into intermediate cells. The intermediate compartment is divided between the basal and suprabasal/secretory cell layers [van Leenders et al., 2003; Uzgare and Isaacs, 2004]. When visualized by staining with Ki-67/MIB1, proliferative cells are observed along the basement membrane and daughter cells move into the suprabasal layer. During this transition, cells experience the greatest change in integrin expression. As cells lose substratum adhesion, integrin expression diminishes. Most notably is the loss in β4 integrin expression, resulting in an increase in α6β1 [Cress et al., 1995]. There is also a concomitant decrease in expression of the other β1 integrins, α3β1 and α2β1. A similar loss in β4 integrin expression occurs during keratinocyte differentiation, which can be triggered by PKCδ activation or Myc expression [Gandarillas and Watt, 1997; Alt et al., 2001; Gebhardt et al., 2006]. It is uncertain whether the loss of integrin expression is caused by the gain in AR expression, since in prostate cancer cell lines forced expression of AR reduces expression of β4 and other integrins [Bonaccorsi et al., 2000; Nagakawa et al., 2004]. Thus, it is conceivable that adhesion to basement membrane exerts a negative regulatory effect on AR expression and that the loss of cell adhesion is a requirement for AR protein expression in the normal prostate epithelium.

The intermediate compartment expands in atrophic glands and in a common condition by the name of proliferative inflammatory atrophy (PIA). Characteristically in PIA, intermediate cells in the suprabasal layer label with Ki-67/MIB1 [van Leenders et al., 2003]. However, it is unclear whether the Ki-67 positivity reflects G0/G1 and G1/S phase progression in the suprabasal layer, or whether cell-cycle entry is initiated in the basal layer and cells move suprabasally during cytokinesis in G2/M maintaining Ki-67 expression. It is conceivable that because these cells do not "sense" a substratum, they fail to re-express integrins in the subrabasal layer after cell division. In the suprabasal layer, intermediate cells express a low level of nuclear AR. We postulate that in suprabasal intermediate cells, androgen stimulates differentiation and not proliferation, since as AR expression increases, proliferation declines. Thus, androgen is not the driving force for expansion of this compartment in PIA. This is consistent with the inhibition of cell growth by androgen in AR-expressing cultured primary epithelial cells, which are of the basal and intermediate phenotype [Robinson et al., 1998; Fry et al., 2000; Lang et al., 2001; Berger et al., 2004]. Histologic inspection and immunohistochemical visualization of AR protein in normal prostate epithelium clearly demonstrates that adhesion to the substratum and expression of AR occurs in distinct cell types and distinct epithelial cell layers. While cell adhesion receptors regulate the proliferation of intermediate cells that are attached to the substratum, androgen and AR likely mediate growth arrest and differentiation in suprabasal intermediate cells as they begin to further differentiate into secretory cells.

Secretory Epithelial Compartment

The differentiated secretory cells in the mouse and human prostate are terminally differentiated and post-mitotic, and thus in these cells AR does not stimulate cell proliferation but regulates the synthesis and secretion of proteins. The effects of androgen on epithelial homeostasis have been primarily explored in rodent prostate. Enforced hyper-expression of AR in otherwise normal mouse secretory epithelium does not stimulate proliferation suggesting androgen action is likely necessary for cell viability and differentiation [Han et al., 2005]. Indeed, in adult mice, castration leads to massive apoptosis of secretory epithelial cells within 48–72 h. Secretory epithelial apoptosis in castrated mice is attributed to the decline of paracrine stromal factors. While the mouse epithelium contains sparse basal cells, the human gland is lined by a continuous basal cell layer, a barrier that potentially shields secretory cells from stromal factors. Thus in mice, androgen-regulated stromal factors have easy access to secretory cells, whereas this is not the case in the human gland. Because of this difference stromal-epithelial interactions may not be the same in mouse and human prostate. In fact, careful inspection along prostatic ducts in rodent prostate revealed less apoptosis in the proximal, basal cell-rich region compared to the tips of the ducts. Based on this observation a barrier function of the basal epithelial layer was suggested [Tenniswood et al., 1992].

Upon androgen suppression in patients, secretory cell numbers diminish. However, focal areas of viable epithelium may persist even after 6–9 months of treatment, indicating that the prostate tissue can maintain an elevated androgen level even when serum androgen is markedly decreased, or that androgen may not directly support the viability of secretory epithelial cells. It is unclear whether secretory cells die because of a change in paracrine factors from the stroma or because of loss of intrinsic AR activity.

In summary (Table I), in the normal human adult prostate epithelium cell adhesion to the substratum and expression of AR occur separately in the basal and suprabasal-luminal cell layers, respectively. Thus, in normal epithelium, signaling pathways from cell adhesion and androgen stimulation do not interact. While adhesion to the substratum facilitates the transduction of stromal signals and mediates cell proliferation and survival, androgen primarily causes protein secretion and might maintain the viability of luminal prostate epithelial cells.

CHANGES IN SUBSTRATUM ADHESION, GFR EXPRESSION, AND ANDROGEN RESPONSIVENESS DURING PROSTATE CANCER DEVELOPMENT

Tissue Analysis of Prostate Cancer Development and Androgen Responsiveness

The development of invasive prostate cancer occurs through an intermediary in situ carcinoma stage, which is referred to as prostatic intra-epithelial neoplasia (PIN). In the early stage of PIN, AR expressing carcinoma cells

reside above a continuous basal cell layer. As PIN progresses, the basal cells disappear and carcinoma cells adhere directly to the substratum (see Table I). Theories for the loss of basal cells include overgrowth of carcinoma cells, invasion of carcinoma cells into the basal cell layer, and apoptosis of basal cells [Bonkhoff, 1996; Yu et al., 2004]. As prostate cancer invades, AR expressing tumor cells interact through integrins with the substratum. Coincidently, the androgen-axis stimulates cell proliferation and survival, in addition to protein secretion. Therefore, de novo adhesion of prostate cancer cells to the substratum may regulate the activity of the AR in prostate cancer cells. This is strikingly different to normal epithelium where substratum adhesion and growth factor activation are spatially separated from AR expression into two different cell layers. Thus, we propose that the switch in AR function to promote proliferation and survival in cancer cells, as opposed to growth suppression and differentiation in normal cells, is facilitated by the interaction of cancer cells with the substratum and the integration of downstream signaling pathways from integrins, growth factors receptors, and AR. We will present examples in a later section to illustrate how cancer cells integrate the downstream pathways from these three signals.

Androgens have a marked effect on prostate cancer cell proliferation and viability in vivo. When patients with androgen-sensitive metastatic disease are androgen ablated the proliferation of cancer cells in the prostate 5 significantly inhibited and massive numbers of cancer cells eventually die. In addition, the proliferation of cancer cells in the prostate is significantly inhibited by anti-androgenic therapy and cancer cells eventually die [Reuter, 1997]. During prolonged androgen suppression, the cytoplasm of cancer cells and of normal secretory cells becomes vacuolated and the nuclei are irregular and mildly pyknotic. These histological features, in addition to the long duration before cell death, are suggestive of autophagy and not of apoptosis. Thus, in contrast to basal cells, adhesion to the substratum is not sufficient for survival of androgen-dependent prostate cancer cells and in the absence of androgens, cancer cells stop proliferating and eventually die. However, an androgen independent population of cancer cells may arise, whose survival is no longer dependent on androgen. This likely occurs through the acquisition of additional oncogenic events that reduce the androgen requirement for activation of AR. It is likely that integrins play an important role in the progression to androgen-independent disease because they augment the activity of kinases that phosphorylate and activate the AR under reduced androgen concentrations. The emerging cells may have a greater dependence on cell adhesion to the substratum for survival, compared to androgen-dependent tumors, and use the substratum to regulate GFR and AR signaling to enhance cell survival.

Integrins and Extracellular Matrix Proteins in Locally Invasive Prostate Cancer

Invasive prostate cancer glands in humans are lined by a single layer of tumor cells. The cancer cells retain certain properties of basal cells, but also express markers of secretory cells including cytokeratin 8 and 18, AR, and PSA. The observation that cancer cells are differentiated according to cytokeratin 8 and 18 expression and positivity for AR and PSA, but negative for basal cell markers, p63, keratin 5 or 14. The observation that cancer cells coexpress basal and secretory cell markers prompted a model in which oncogenic transformation occurs within the intermediate compartment and triggers an aberrant differentiation program. As a result, we would expect that GFRs normally expressed on basal and not on secretory cells remain expressed in some cancer cells. Indeed, several GFRs are noticeably elevated in prostate cancer cells compared to secretory cells [Ware, 1998; Knudsen et al., 2002].

However, because cancer cells are more differentiated than basal cells and because cancerous glands lack a basal cell layer, we anticipate differences in cell adhesion complexes as well as substratum constituents between normal epithelium and cancer. Our insight into integrin expression and substratum composition is based on a detailed immunohistochemical analysis in frozen tissues [Knox et al., 1994; Cress et al., 1995]. The substratum of tumor glands, compared to normal glands, is altered. Specifically, laminin 5 and collagen VII are lost in cancer, but laminin 10/11 and collagen IV are retained. This alteration directly correlates with the loss of the laminin 5 binding integrins, $\alpha6\beta4$, and the reduced expression of $\alpha3\beta1$ on cancer cells. The prostate cancer integrin, $\alpha6\beta1$, engages laminin 10/11 [Cress et al., 1995]. Compared to the normal epithelium, two addi-

tional differences in integrin expression exist in the carcinoma cells: the β1C integrin splice-variant shifts to β1A and a truncated α6 variant, α6p, is abundantly expressed [Fornaro et al., 2000; Demetriou et al., 2004]. The changes in integrin and substratum protein expression are likely to be important in tumor development. For instance integrin β1A stimulates proliferation in vitro [Goel et al., 2005], while α6p integrin triggers invasion [Rabinovitz et al., 1995]. Thus, signaling specifically through laminin 10/11 and an α6(p)β1A integrin variant may enhance tumorigenesis. In addition it is likely that intracellular changes in signal transduction pathways accompany α6(p)β1A expression. Whether or not these affect AR function remains to be investigated.

The α6p variant may also be important in the development of metastatic cancer. α6p is generated by cleavage of its extracellular domain by the extracellular protease uPAR. It lacks the ligand-binding domain and therefore no longer interacts with the substratum [Demetriou et al., 2004]. The result would be decreased adhesion and increased mobility in tissues [Blasi and Carmeliet, 2002]. Interestingly, the tetraspanin CD82, which is an α6 integrin-interacting protein and a metastasis suppressor gene, suppresses uPAR activity [Bass et al., 2005]. Thus, loss of CD82 expression during tumor progression may be one of the reasons for increased uPAR activity and cleavage of α6 integrin.

AR, Integrins, and GFRs in Locally Invasive Prostate Cancer

When expressed in basal epithelial cultures or in the PC3 cancer cell line, AR suppresses cell proliferation, while in xenografts of most prostate cancer cell lines, and in prostate cancer in vivo, androgen stimulates cell proliferation [Heisler et al., 1997; Berger et al., 2004]. Therefore, there may be a disconnect between AR and androgen action. In transgenic mice, a single point mutation in AR was sufficient to trigger tumor development and progression while enforced hyper-expression of wild-type AR was not [Han et al., 2005]. On the other hand, oncogenic immortalization of normal human prostate epithelial cells and co-expression of wild-type AR was sufficient to induce androgen-dependent tumors in a xenograft model [Berger et al., 2004]. In these tumors androgen was necessary for cell proliferation. The mutationally activated AR alone is sufficient to induce prostate cancer in mice; however, in human cancer AR mutations occur late in oncogenesis and are not the cause for prostate cancer development. Together these results suggest that the proliferative activity of the AR is context dependent and requires oncogenic transformation. It is likely that oncogene-induced enhanced expression and activation of GFRs and integrins are required to increase the proliferative activity of the AR.

Evidence for cooperation between integrins and GFRs for regulating cell proliferation has been well documented [Miranti and Brugge, 2002]. Integrin crosstalk with IGFR1 is required for efficient IGF-1 signaling [Walker et al., 2002; Clemmons and Maile, 2005]. In return, IGF-1 enhances integrin-mediated adhesion and spreading [Hermanto et al., 2002]. Recent studies have demonstrated that IGFR is critical for prostate cancer development [Wu et al., 2005a] and transgenic mice expressing high levels of IGF-1 under control of the K5 promoter develop prostate tumors [DiGiovanni et al., 2000]. IGFR1 forms complexes with α6 integrin and recruits activated MAPK [Walker et al., 2002]. Thus, integrin α6β1-laminin 10/11 interactions may cooperate with IGF/IGFR to promote the early development of prostate cancer. Interestingly, the β1A integrin variant, which is increased in prostate cancer, promotes IGF-1-mediated cell proliferation, while the β1C integrin variant, which is downregulated in prostate cancer, inhibits IGF-mediate proliferation [Goel et al., 2005]. A monoclonal antibody to IGFR1 inhibits tumor proliferation in both AR-dependent and AR-independent models of LUCap35 prostate cancer xenografts, suggesting that androgen and IGFR independently support proliferation [Wu et al., 2005a]. Future research will need to address which oncogenic events cause a switch in AR activity from inhibition to stimulation of cell proliferation, and determine whether cancer specific cell adhesion to basement membrane or expression of GFRs contribute to the switch in AR activity.

AR, INTEGRINS, AND GFRS IN THE REGULATION OF CELL PROLIFERATION AND SURVIVAL IN METASTATIC PROSTATE CANCER

Integrins and Extracellular Matrix in Metastatic Prostate Cancer

Understanding of the role of integrins in prostate cancer metastasis has been stymied

by the lack of in vivo immunohistochemical data in metastatic tissues and thus our current knowledge is derived from analyzing cell lines [Fornaro et al., 2001]. Cellular models of prostate cancer progression have largely been derived from two cell lines, LNCaP and PC3. In these models integrin expression, utilization, and function have been studied; however, it is uncertain whether in vivo correlates exist. The LNCaP cells are androgen-sensitive and thus recapitulate characteristics of androgen-responsive primary prostate cancer cells. One might therefore expect that LnCaP cells express a repertoire of integrins that is similar to primary prostate cancer. However, contrary to primary prostate cancer tissues, LNCaP cells express low levels of the laminin receptors α6β1 and α3β1, but high levels of the fibronectin receptor, α5β1 [Witkowski et al., 1993; Edlund et al., 2001]. PC3 and DU145 cells, derived from metastatic lesions, do not express AR and express elevated levels of α5β1, as well as the vitronectin receptor, αvβ3, neither of which have been reported to be expressed in vivo [Cooper et al., 2002]. αvβ3 expression is typically not seen in normal epithelial cells, although one report suggests that it is expressed in primary tumors [Zheng et al., 1999]. Whether α5β1 or αvβ3 are expressed in metastatic tumors in vivo has not been demonstrated. In cell lines, expression of integrins α5β1 and αvβ3 might be caused by high fibronectin and vitronectin levels in serum used for cell culture. The selection pressure during the establishment of cells lines could favor cells that upregulate fibronectin- and vitronectin-binding integrins. Changes in integrin expression and function during prostate cancer progression and metastasis formation might be an important contributing factor to tumor growth and development of treatment resistance.

The role of β4 integrin in metastatic prostate cancer remains controversial. Elevated levels of β4 integrin have been routinely observed in primary and metastatic breast and colon cancers [Natali et al., 1992; Davis et al., 2001]. However, it is not expressed in primary prostate cancers in vivo. Metastatic prostate cancer cell lines express β4 integrin, but it is not known whether β4 is expressed in prostate metastases in vivo. Since forced expression of AR in metastatic cell lines decreases expression of β4 integrin, β4 expression in cell lines may simply indicate the loss of the androgen/AR signaling axis due to in vitro culturing [Bonaccorsi et al., 2000; Evangelou et al., 2002; Nagakawa et al., 2004]. However, β4 integrin expression in metastatic cells could serve a different function than in basal epithelial cells, since metastatic cells do not form hemidesmosomal structures and fail to deposit laminin 5. If β4 integrin expression in metastatic prostate cancer cell lines recapitulates integrin expression in prostate cancer metastasis in vivo and is not an artifact of cell culture, then re-expression of β4 in androgen-independent tumors may play a unique role in prostate cancer metastasis. Further studies will be necessary to validate the role of β4 integrin in metastasis.

In addition to the reported changes in substratum in primary prostate cancer, it is expected that metastatic cells will see an even different substratum. Over 80% of prostate metastases are found in the bone. Collagen I is one of the primary substratum proteins in the bone and α2β1 integrin is primarily responsible for adhesion to collagens. While basal prostate epithelial cells express α2β1 and bind collagen IV in the basement membrane [Knox et al., 1994], there is significantly less, but measurable expression of α2β1 in primary prostate cancers. The metastatic cell lines express α2β1 at levels similar to basal cells, with PC3 cells showing slightly higher levels of expression. Treatment of PC3 cells with the bone-derived growth factor, TGF-β1 increases α2β1 levels as well as adhesion and spreading [Kostenuik et al., 1997]. Thus, signaling through collagen/ TGF-β1 in the bone environment may favor metastatic growth in part through increasing integrin engagement. How signaling through TGFβ1 and α2β1 impacts AR function in the metastatic cells is unknown.

Integrin expression and function can be modulated by interactions with other proteins, which may be important in metastasis. CD82/ KAI1 was identified as a metastasis suppressor of prostate cancer cells in a metastasis screen in rats [Dong et al., 1996]. CD82 is a tetraspanin that controls the activity of the prostate cancer integrins, α6β1 and α3β1 [Maecker et al., 1997] as well as negatively regulates EGFR and c-Met [Jackson et al., 2003; Odintsova et al., 2003; Sridhar and Miranti, 2005]. CD82 exerts its effects by limiting the distribution and association of integrins and GFRs on the cell surface. Thus, in advanced stages of prostate cancer reduced expression of CD82 as it is documented

to occur in vivo would permit associations and redistribution of integrins and GFRs leading to enhanced signaling and an augmentation of cell proliferation and androgen insensitivity.

Crosstalk Between Integrins, AR, and GFRs in Metastatic Prostate Cancer

AR is considered the main culprit of metastatic growth and treatment failure [Grossmann et al., 2001]. Although metastatic tumors are androgen independent, they still rely heavily on AR for growth and survival [Feldman and Feldman, 2001]. Signaling pathways emanating from GFRs and integrins may reduce the dependence on androgen and augment the transcriptional activity of AR in the progression to androgen independent disease. From studies in cell culture models, several mechanisms for interactions between integrins, GFRs, and AR have been reported and can be grouped into four paradigms.

Paradigm 1: Signal transduction pathways from integrins and GFRs activate kinases that affect the expression and activity of AR and AR-coregulators through phosphorylation. Enhanced signaling through GFRs is thought to play an important role in enhancing AR activity, especially in the progression to androgen independence. The crosstalk between integrins and GFRs also intensifies during prostate cancer progression particularly if GFRs are over expressed. For example, at normal expression levels, engagement of integrins activates the EGFR kinase, but this is not sufficient to induce cell proliferation. However, over expression of EGFR, permits cell-cycle progression through integrin engagement [Bill et al., 2004]. Several studies have attempted to address whether members of the EGFR family, including ErbB2/Her2/Neu, are significantly over expressed in prostate cancer metastases. So far there is limited evidence to support ubiquitous over expression [Ware, 1998]. In contrast to the EGFR family, c-Met is expressed in practically all metastatic prostate cancers and is significantly over expressed in prostate bone metastases compared to soft tissue metastasis [Knudsen et al., 2002]. Its loss in PC3 cells results in apoptotic cell death [Shinomiya et al., 2004] and c-Met could therefore be a driver of metastatic growth and tumor cell survival. A recent study demonstrated a shift from paracrine growth stimulation of the androgen-dependent CWR22 xenograft model to autocrine growth stimulation through hepatocyte growth factor (HGF) secretion in the androgen-independent xenograft [Nakashiro et al., 2004]. In patients, the c-Met ligand, HGF, is secreted by osteoblasts and thus paracrine activation of c-Met could occur in metastatic prostate cancer cells in the bone. However, if c-Met is sufficiently over expressed, activation may be integrin and not HGF dependent [Wang et al., 2001]. Finally, the observation that loss of the integrin binding protein CD82 enhances c-Met activation by both integrins and ligand and CD82 loss correlates with poor prognosis and metastatic disease further supports the potential importance of c-Met in prostate cancer metastasis [Sridhar and Miranti, 2005]. Thus, c-Met is a candidate GFR that through crosstalk with integrins might activate cytoplasmic kinases that phosphorylate AR.

Multiple cytoplasmic kinases, including PKA, PKC, and MAPK phosphorylate AR in its N-terminal domain [Alt et al., 2001; Gioeli et al., 2002] (Fig. 1B). These kinases have been reported to cause AR activation downstream of cell surface receptors for IGF-1, KGF, EGF or downstream of ErbB2, even when androgen concentrations are low [Grossmann et al., 2001; Culig et al., 2002; Chatterjee, 2003; Rahman et al., 2004]. However, it is uncertain whether kinase activation always permits a proliferative function of AR. In two separate studies increased expression of active MAPK was noted in the center of prostate cancers or in areas of increased cancer cell proliferation [Gioeli et al., 1999; Paweletz et al., 2001]. Therefore, phosphorylated MAPK can provide a signal for differentiation [Gmyrek et al., 2001] (central region) or proliferation (high grade cancer), and it is conceivable that the MAPK signal is in part propagated through phosphorylation of AR. Thus, the phosphorylated AR might stimulate differentiation or proliferation dependent on the underlying spectrum of oncogenic changes in the cancer. Based on preclinical studies in LNCaP cells demonstrating that knockdown of AR causes cell-cycle arrest, and under some conditions apoptosis, in LNCaP cells [Zegarra-Moro et al., 2002; Liao et al., 2005; Yang et al., 2005], eliminating AR protein expression as well as inhibiting the Ras/MAPK pathway are currently being considered as therapeutic approaches. However, it may be necessary to thoroughly evaluate the activity of the AR and MAPK throughout the cancer and avoid using

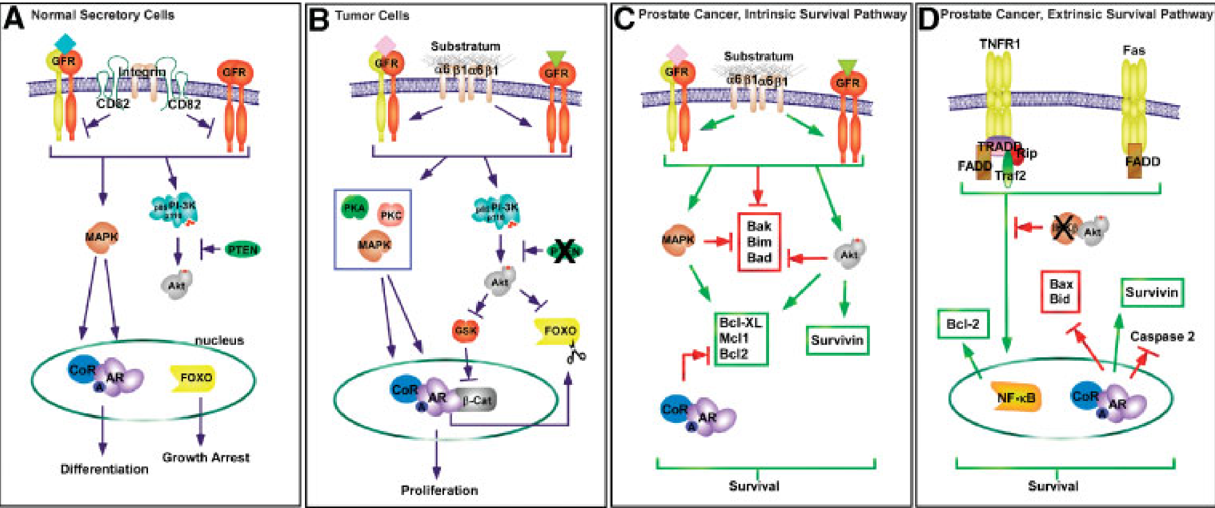

Fig. 1. Convergence of signal transduction pathways from integrins, cell surface receptors, and the androgen receptor regulate differentiation, proliferation, and survival. **Panel A**: Normal secretory epithelium. The expression of growth factor receptors (GFR) and integrins in secretory epithelial cells is low compared to basal epithelial cells or prostate cancer cells. Secretory cells do not directly contact the substratum. CD82 is expressed and limits the activity of GFRs. The Akt pathway is not significantly activated, since PTEN is present, and the FOXO proteins are in the nucleus, inhibiting cell proliferation. It is conceivable that MAPK is activated, since sustained MAPK activation stimulates cellular differentiation. In this case, active MAPK might phosphorylate the AR and AR co-regulators (CoR). The AR is in the nucleus and androgens induce and maintain a differentiation phenotype. Secretory cells are post-mitotic and it is unclear which proteins and pathways are responsible for their survival. **Panel B**: Prostate cancer cells. Prostate cancer cells express integrins α6β1 (or α3β1). The loss of CD82 permits interaction of integrins with growth factor receptors (GFR), leading to their activation and induction of signal transduction pathways. The convergence of signals from integrins and GFRs regulates cytoplasmic kinases (PKA, PKC, and MAPK), which phosphorylate AR and AR co-regulators. Phosphorylation regulates interactions between the AR and AR binding protein as well as interactions between AR and other transcription factors. The PI3K/Akt pathway plays a central role in proliferation and survival of prostate cancer cells, in part through regulating the activity of FOXO transcription factors. Akt phosphorylates FOXO proteins, sequesters them in the cytoplasm and thereby inhibits their anti-proliferative activity. FOXO proteins are cleaved by an androgen-induced protease and this may contribute to the proliferative effects of androgen in prostate cancer cells. In addition, Akt inhibits GSK3β, which stabilizes β-catenin and leads to its enhanced expression. Nuclear translocation of β-catenin may be assisted by binding to the AR and in the nucleus β-catenin stimulates cellular proliferation. **Panel C**: The intrinsic apoptotic pathway in prostate cancer cells. Integrins, growth factor receptors (GFR), and AR regulate expression of Bcl-2 and BH3 family proteins. MAPK and Akt induce expression (green) of prosurvival proteins, Bcl-2, Bcl-XL, and Mcl-1, while decreasing expression (red) of Bad, Bak, and Bim. The expression of the prosurvival protein, Survivin is also upregulated by Akt. AR suppresses transcription of Bcl-2. The balance between integrin and GFR positive signals and AR-driven negative signals determine cell fate. **Panel D**: The extrinsic apoptotic pathway in prostate cancer cells. Cell surface receptors (TNFR1, Fas) that stimulate apoptosis limit the viability of prostate cancer cells through regulation of NF-κB. Nuclear translocation of NF-κB is tightly regulated and in the nucleus where it stimulates Bcl-2 expression. Nuclear translocation of NF-κB is inhibited through binding to phosphorylated IκB. IκB is phosphorylated by IκB kinase (IKK), which is phosphorylated by Akt and targeted for degradation. Thus, Akt activation causes nuclear translocation of NF-κB. AR can inhibit apoptosis by suppressing the transcription of caspase 2 through an androgen receptor-binding element in the promoter of caspase 2.

these therapies for cancers in which AR or MAPK primarily cause cell differentiation.

Another probable mechanism for cooperation between AR, integrins, and GFRs is through transcriptional coregulators. Progression to androgen independence is associated with changes in AR coactivator expression. Coactivators enhance AR function by bridging to non-androgen regulated transcription factors and thereby connecting androgen dependent and independent pathways. While much in vitro data have linked coactivators to enhanced signaling by AR, evidence that this occurs in vivo is still lacking. The AR coactivators, ARA70, ARA55, and ARA54, are over expressed during androgen ablation and in androgen insensitive tumors [Culig et al., 2002; Chatterjee, 2003]. Over expression of either coactivator increases the sensitivity to androgens, anti-androgens, and estrogens. SRC-3 and ARA70 interactions with AR are enhanced by their phosphorylation through ErbB2/EGFR and activation of MAPK [Heinlein and Chang, 2004]. ErbB2 also stimulates PI3K (probably as a dimer with ErbB3), which leads to phosphorylation of AR on Ser213/791 [Culig et al.,

2005], however, the importance of these phosphorylation sites for AR activity is debatable. The AR coactivator, ARA55, takes a unique position, since its phosphorylation by the integrin-regulated kinase Pyk2 blocks its binding to AR [Heinlein and Chang, 2004]. Thus, the combination of coactivator and AR over expression and the regulation of interactions by integrin-activated signaling pathways may provide the underlying cause for the switch of a growth inhibitory to a growth stimulatory effect of the androgen axis during prostate cancer development.

Paradigm 2: Integrins and androgen cooperatively promote cell survival by regulating the Akt pathway and the expression of pro-survival Bcl-2 family proteins and Survivin.

Androgen/AR and regulation of the Akt pathway. The most frequently affected and best-studied survival pathway in prostate carcinoma is the PI3K/Akt pathway. In cancer cells, Akt is activated by integrins, growth factors, and through loss of the tumor suppressor, PTEN. In human samples, increased Akt activity correlates with advanced disease and high Gleason score [Ghosh et al., 2003], is an adverse prognostic indicator [Ayala et al., 2004; Kreisberg et al., 2004], and increases the danger of cancer recurrence [Thomas et al., 2004]. Conditional loss of both alleles of PTEN in AR-expressing epithelial cells is sufficient to induce prostate cancer in mice [Wang et al., 2003]. In prostate cancer cells with intact PTEN expression the Akt pathway is activated by GFRs. For instance, IGFR or ErbB2/ErbB3 mediates activation of the PI3K/Akt pathway which leads to increased activity of AR [Heinlein and Chang, 2004]. In prostate cancer cells, activation of IGFR by its ligand IGF-1 is increased through elevated IGFBP-5 and decreased through diminished IGFBP-3 expression [Culig et al., 2002]. Thus, even in tumors where PTEN is intact, signaling by growth factors could stimulate survival through PI3K/Akt.

Another mechanism for activating the Akt pathway is through integrins (Fig. 1B). Integrins are essential co-receptors for growth factor-mediated activation of the Akt pathway [Cabodi et al., 2004]; however, there is also evidence that integrins can activate the Akt pathway through GFRs, independent of growth factors [Moro et al., 1998]. Cells prevented from adhering to the substratum undergo a form of apoptotic cell death termed anoikis [Reddig and Juliano, 2005], and in some cell types adhesion-induced cell survival depends on PI3K [Frisch and Screaton, 2001]. However, PI3K/Akt is not always responsible for adhesion-mediated cell survival, and in particular adhesion of basal cells to laminin 5 does not significantly activate PI3K, neither does blocking PI3K lead to cell death [Lin et al., 1999; Uzgare and Isaacs, 2004]. In cultured basal prostate epithelial cells, cell survival on laminin 5 requires signaling from $\alpha3\beta1$ integrin via EGFR to active the Ras/MAPK pathway (Miranti, unpublished data) [Manohar et al., 2004]. Oddly, death of primary prostate epithelial cells induced by loss of $\alpha3\beta1$/laminin 5 signaling does not occur through the classical intrinsic apoptosis pathway, contrary to tumor cells which die through activation of classical apoptosis pathways [Uzgare and Isaacs, 2004]. Thus, during tumor development loss of laminin 5 and $\alpha3\beta1$, or dependence on $\alpha6\beta1$ and laminin 10/11 signaling may alter adhesion activated cell survival pathways. Changes in cell adhesion, activation of growth factors receptors, and PTEN loss may all exert selective pressures on cancer cells that affect their dependence on androgen as a survival factor.

While activation of the PI3K/Akt pathway is critical for survival, its inhibition is not sufficient to cause death of cancer cells and can be rescued by androgen or growth factors. Thus, in addition to inhibiting the PI3K pathway, the removal of androgens or growth factors is required for inducing apoptosis [Carson et al., 1999; Lin et al., 1999; Murillo et al., 2001] and both PI3K-dependent and -independent survival pathways operate in prostate cancer cells to maintain viability [Carson et al., 1999]. This has important implications for therapeutic strategies, as simply inhibiting Akt would not be cytotoxic for cancer cells and cause tumor regression.

Androgen/AR and regulation of extrinsic and intrinsic apoptotic pathways. Whether a cell lives or dies is in part determined by the activity of the extrinsic and intrinsic apoptotic pathways. While the extrinsic apoptotic pathway signals downstream of death cell surface receptors, the intrinsic pathway is regulated through expression of Bcl-2 family members (Fig. 1C,D). Both pathways interact with the androgen/AR axis. Intrinsic apoptosis is driven by Bax and Bak [Wei et al., 2001]. Bax and Bak are antagonized by three Bcl-2-family members, Bcl-2, Bcl-XL, and Mcl-1 [Gelinas and White,

2005] and increased expression of all three has been noted in mid to late stage prostate cancer [Krajewska et al., 1996]. Since Bcl-2 expression in prostate cancer is associated with tumor progression, its expression level is of keen importance and is regulated by androgen, growth factors, and integrin expression.

Upon GFR activation, the Akt pathway may be synergistic with Bcl-2 for cell survival [Huang et al., 2001]. In addition to Bcl-2, Bcl-XL may also assume an important role in supporting cell viability. In PC3 and LNCaP cells, Bcl-XL sustains survival when the PI3K pathway is inhibited [Yang et al., 2003]. There is clear antagonism between androgens and Bcl-2. Evidently in vivo, androgens suppress Bcl-2 transcription and androgen ablation upregulates Bcl-2 [Huang et al., 2004b]. Furthermore, the ability of Bcl-2 to enhance cancer growth only occurs in androgen-depleted conditions. Thus, increased Bcl-2 expression might be a requirement for progression to androgen independence [Grossmann et al., 2001]. Thus, both Bcl-2 and Bcl-XL appear to be important for survival of prostate carcinoma cells in the absence of androgens or in low androgen conditions.

In androgen-dependent cells, TNF-α activation normally induces cell death through the extrinsic cell death pathway. Surprisingly, upon removal of androgens and sustained Akt pathway activation, TNFα stimulates cell survival [Catz and Johnson, 2003]. This response requires the degradation IKK, which permits nuclear translocation of NF-κB and increased Bcl-2 transcription (Fig. 1D). Since the NF-κB pathway is suppressed by androgens, this may explain the increase in Bcl-2 expression upon androgen ablation.

Integrins stimulate cell survival through upregulation of Bcl-2 proteins and through inhibition of proapoptotic proteins such as Bim, Bad, and Bak [Zhang et al., 1995] (Fig. 1C). Studies in our lab with basal prostate epithelial cells and in other labs with keratinocytes indicate that adhesion to laminin-5 regulates cell survival through α3β1 integrin-mediated activation of the Ras/MAPK pathway [Ryan et al., 1999; DiPersio et al., 2000]. We further noted upregulation of Bcl-XL and the downregulation of Bim under the same conditions (Miranti, unpublished data). Thus, the changes in integrin expression during prostate cancer progression may regulate cell survival through Bcl-XL expression.

In vivo androgens clearly regulate tumor cell survival, however, how AR interacts with the intrinsic or extrinsic pathways is largely unknown (Fig. 1C,D). The recent discovery of a functional ARE in the caspase 2 gene and its inhibition by androgen, suggests that this may be a mechanism by which androgens directly regulate apoptosis [Rokhlin et al., 2005]. Decreased expression of caspase 2 was sufficient to prevent TNFα- or TRAIL-induced apoptosis. Androgens can also mediate cell survival by inducing expression of Survivin. Survivin is an anti-apoptotic protein that blocks caspase activity [Goel et al., 2005]. In a recent study in metastatic PC3 cells, adhesion to fibronectin was found to upregulate Survivin levels. This was dependent on PI3K and responsible for inhibiting TNFα-induced apoptosis [Fornaro et al., 2003]. Thus androgens, GFRs, and integrins all regulate many of the same molecules and pathways that are important for survival of both normal and tumor cells. The relative intensity of signals from each pathway, the presence of oncogenic mutations, and the extent of crosstalk will determine which pathways predominate and likely guide tumor progression.

Paradigm 3: AR interacts with transcription factors that are activated at the end of signal transduction pathways. One possible explanation for the interdependence of PI3K and AR in promoting cell survival is that they cooperate to reduce the activity of forkhead box-O transcription factors (FOXO). Translocation of FOXO transcription factors into the nucleus triggers growth arrest and apoptosis (Fig. 1A). Akt inhibits FOXO proteins by direct phosphorylation, which causes their sequestration in the cytoplasm [Greer and Brunet, 2005]. Under certain conditions, AR may bind FOXO1 and inhibit nuclear entry [Li et al., 2003]. In addition, androgen induces a cysteine protease that cleaves and inactivates FOXO1 [Huang et al., 2004a]. An important cell proliferation target of FOXO proteins is the Cdk2 inhibitor, p27kip [Lynch et al., 2005] whose loss of expression is associated with prostate cancer development in mice and adverse patient outcome [Di Cristofano et al., 2001].

The recent finding that β-catenin shuttles with AR into the nucleus and is found in AR transcriptional complexes, suggests that the Wnt signaling pathway interacts with AR [Song and Gelmann, 2005; Verras and Sun, 2005]. Akt phosphorylation of GSK3β further enhances

β-catenin/AR interactions by stabilizing the cytoplasmic expression of β-catenin (Fig. 1B) [Sharma et al., 2002; Mulholland et al., 2006]. Whether this contributes to androgen-dependent cell proliferation or survival has not been determined.

Paradigm 4: Androgen/AR and AR-coregulators regulate the expression and activity of growth factors and growth factor receptors. Androgen increases expression of GFRs such as EGFR or growth factors such as KGF, IGF, EGF, TGFα, or VEGF through enhancing the stability of mRNA expression or through increases in gene transcription via AR co-activators [Wu et al., 2005b]. Thus, GFRs could be involved in an autocrine loop to perpetuate the activity of AR. In addition, it is plausible that androgens, like estrogens or glucocorticoids, regulate the stability of integrins [Ing, 2005].

Androgens not only stimulate the release of paracrine stromal factors, but may also regulate their activation. We demonstrated that androgen suppression causes decreased expression of hepatocyte activator inhibitor (HAI-1) in basal and intermediate cells of normal prostate epithelium [Knudsen et al., 2005]. HAI-1, a transmembrane serine protease inhibitor, is activated by androgen-stimulated cleavage from the cell surface [Martin et al., 2004]. It is an inhibitor of Matriptase and Hepsin, which is over expressed in human prostate cancer metastasis and drives prostate metastasis to mouse bone [Dhanasekaran et al., 2001; Oberst et al., 2001; Klezovitch et al., 2004; Herter et al., 2005]. HGF, the ligand for the c-Met receptor, is activated through proteolytic cleavage by Matriptase/Urokinase and as discovered recently, also by Hepsin [Herter et al., 2005; Kirchhofer et al., 2005]. Thus, HGF activity is regulated by androgen through HAI-1. Thus, through regulating the activation and localization of HAI-1, androgen indirectly modulates the activity of the HGF/c-Met axis.

THERAPEUTIC OPPORTUNITIES BASED ON ANDROGEN-REGULATED GROWTH FACTOR EXPRESSION AND CELL ADHESION IN PROSTATE CANCER

Combination Therapies With Androgen Ablative Treatment

An attractive conceptual approach for treating advanced prostate cancer is to administer androgen-ablative treatment, and to simultaneously target pro-survival proteins that are upregulated as a consequence of androgen deficiency. In addition to Bcl-2, the expression of Clusterin, Hsp 27 and IGFBP-2 and -5 increases in prostate cancer cells upon androgen suppression. Interestingly, these proteins strengthen cell adhesion. The increased cell adhesion may provide a substantial survival impulse and reduce the dependence on androgen for viability. Under conditions of stress, such as during androgen deficiency, chemotherapy, and radiation therapy, cancer cells survive through upregulation of cell adhesion pathways that when targeted lead to their death.

The secreted form of Clusterin is glycosylated, deposited in the extracellular matrix, and affects cell adhesion. The precise mechanism by which extracellular Clusterin mediates cell survival has not been elucidated, but a mechanism for intracellular-expressed Clusterin was discovered recently. In preclinical models of prostate cancer, Clusterin antisense improved the efficacy of chemotherapy, radiation, and androgen withdrawal [Miyake et al., 2000]. Hsp27 localizes to focal adhesions, where it binds the AR coregulator ARA55/Hic-5 [Jia et al., 2001]. Various inhibitors affect subcellular localization and phosphorylation of Hsp27, thereby increasing the network of actin stress fibers and numbers of focal adhesions. Thus, an advantage of the Hsp27 antisense strategy is that it may affect cancer cells directly and also inhibits tumor growth through anti-angiogenic activity [Gleave et al., 2005]. IGFBP-5 and IGFBP-2 are two members of the IGFBP family of proteins whose expression increases upon androgen suppression. IGFBP-2 and -5 expression rose in the Shionogi and LNCaP xenografts, as well as in prostate epithelium in vivo when androgen levels are reduced. Our data demonstrate increased expression of IGFBP-5 in castrated mouse bone and bone marrow, suggesting that IGFBP-5 could act as a paracrine growth factor for metastatic prostate cancer cells (Knudsen, unpublished data). Thus, targeting IGFBP-5 would affect both the cancer and its environment. [Chi and Gleave, 2004].

Integrin Targeted Therapies

A humanized monoclonal antibody with specificity for integrin αvβ3 has been tested in clinical trials [Posey et al., 2001; McNeel et al., 2005]. αvβ3 is expressed on sprouting blood

TABLE I. Substratum Adhesion and Response to Androgen Stimulation of Normal Prostate Tissue and of Prostate Cancer

	A-responsive stromal cells	Type of A-responsive epithelial cells	Substratum adhesion of A-responsive epithelial cell	Epithelial response to androgen stimulation	Schematic representation of responsive epithelium	H&E image
Development	Prostate	Basal/intermediate (AR negative)	Yes	Branching morphogenesis	BC	
Adult	Prostate	Secretory (AR positive)	No	Secretion	SC BC StC	
1° Cancer	Prostate	Cancer (AR positive)	Yes	Proliferation, survival, secretion	CC StC	
Metastasis	Bone	Cancer (AR positive)	Yes	Proliferation, survival, secretion	bone CC	

, Proliferating cells; , AR expressing cells; , AR positive nuclei; , AR negative nuclei; BC, basal epithelial cells; SC, secretory epithelial cells; StC, stromal cells; CC, cancer ls.

In human tissues, the effects of androgen are appreciated by contrasting histological and immunohistochemical features in tissues from individuals with normal versus suppressed androgen levels. **Basal epithelial cells** are AR negative. They proliferate during development in response to androgenic factors from the stroma and differentiate into secretory cells. In the adult, androgen suppression results in expansion of the basal cell compartment. **Secretory epithelium** expresses highest AR levels. Androgen stimulates terminal differentiation, growth arrest and protein secretion. Androgen deprivation decreases differentiation and secretion and ultimately induced cell death. **Stromal cells** secrete factors in response to androgen that stimulate ductal development. Branching morphogenesis of epithelium during development requires a coordination of proliferation, differentiation and stromal invasion that is stimulated by androgen-regulated stromal factors. Most **primary prostate cancer cells** require androgen for proliferation, differentiation, survival and secretion. Consequently androgen deprivation in patients

vessels and the rationale for this targeted treatment approach is the inhibition of angiogenesis. The concept for using integrin-directed angiogenesis to inhibit the growth of metastatic prostate cancer is supported in an elegant SCID-human-bone model of prostate cancer bone metastasis [Nemeth et al., 1999]. In this animal model, the growth of PC3 cells implanted in fragments of human bone was inhibited by administration of a human-specific anti-αvβ3. The antibody reduced the growth of human-derived blood vessels and the recruitment of osteoclasts by the tumor [Nemeth et al., 2003]. PC3 cells express preferentially αvβ1 and αvβ5 integrins and an αv siRNA caused an increase in tumor cell apoptosis in PC3 mouse bone xenografts [Bisanz et al., 2005]. Once the integrin repertoire of metastatic prostate cancer cells has been fully characterized, there is hope that additional integrin targets suitable for therapeutic development will be identified. The combined inhibition or cytotoxicity of multiple cell types, including the tumor, will be an effective approach in the treatment of metastatic prostate cancer.

SUMMARY

Cell adhesion to the substratum is a critical cofactor for proliferation and survival of epithelial cells. During the development of prostate cancer, malignant luminal epithelial cells transition from cell–cell adhesion to cell–substratum adhesion. In normal epithelium signals from cell adhesion and AR are separated into different cell layers; however, in cancer cells they are co-expressed. Therefore, the engagement of integrins in prostate cancer cells, namely α6β1 and α3β1 and their prostate cancer variants β1A and α6p, may drastically alter the cells' interpretation of growth factor signals and the activity of the AR. Signals from cell surface integrins and GFRs increase during tumor progression and interact with the AR to modulate its transcriptional activity through AR phosphorylation, AR co-activator regulation, or through regulation of other transcription factors, such as FOXO, β-catenin, and NFκB. These interactions may be responsible for changing the functional activity of the AR from differentiation and secretion in normal epithelium to proliferation and survival in cancer. Despite the notion that cell adhesion is a critical component of prostate cancer progression, there is currently little known about the changes in integrin expression during prostate cancer progression and in metastatic cancer cells. Due to the absence of tissue-based analysis, we can only speculate about the role of integrins in mediating tumor growth and progression to androgen-independent, treatment-refractory prostate cancer. Interestingly, recently identified treatment targets that are upregulated by androgen suppression have an impact on cell adhesion. It is likely that integrin expression increases on the surface of metastatic cancer cells and there maybe forms of integrins that are cancer specific; therefore, these cell surface receptors may constitute promising therapeutic targets. Thus, studying their expression and function in locally invasive and metastatic prostate cancer is critical for the development of better therapeutic approaches against metastatic prostate cancer.

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