Review

Changes in extracellular matrix (ECM) and ECM-associated proteins in the metastatic progression of prostate cancer

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Abstract

Prostate cancer (PCa) is no exception to the multi-step process of metastasis. As PCa progresses, changes occur within the microenvironments of both the malignant cells and their targeted site of metastasis, enabling the necessary responses that result in successful translocation. The majority of patients with progressing prostate cancers develop skeletal metastases. Despite advancing efforts in early detection and management, there remains no effective, long-term cure for metastatic PCa. Therefore, the elucidation of the mechanism of PCa metastasis and preferential establishment of lesions in bone is an intensive area of investigation that promises to generate new targets for therapeutic intervention. This review will survey what is currently know concerning PCa interaction with the extracellular matrix (ECM) and the roles of factors within the tumor and ECM microenvironments that contribute to metastasis. These will be discussed within the context of changes in expression and functional heterodimerization patterns of integrins, changes in ECM expression and reorganization by proteases facilitating invasion. In this context we also provide a brief summary of how growth factors (GFs), cytokines and regulatory signaling pathways favor PCa metastasis to bone.

Background

Prostate cancer (PCa) is the second most common malignancy in men worldwide [1]. In the United States for 2003 there are expected to be 220,900 new diagnoses and approximately 28,900 PCa deaths [2]. Notably, it has been determined that ~90% of patients with advanced PCa will develop osseous metastases [3,4]. Similar studies on PCa patients at autopsy have found ≥ 80% of patients have established macrometastases involving bone, and of these bone lesions ≥ 90% will have an osteoblastic phenotype [5,6]. The characteristics of clinical presentation with bone involvement include severe pain, pathologic fractures and spinal cord compression [7]. Typically these patients have a mean survival time of nine months to one year [8]. Thus, once PCa metastasizes to the bone it is difficult to eradicate.

Metastasis requires the interaction of malignant cells with three distinct microenvironments 1) the primary organ, 2) the circulation, and 3) the target organ where a metastatic lesion will develop [9-11]. Both soluble and insoluble stromal elements within these microenvironments are involved in the metastatic cascade [11]. Successful metastasis requires that several well-documented steps be followed. Initially, angiogenesis must be induced to meet the nutrient needs of the growing cancer cells and facilitate removal of toxic waste products [12,13]. The metastatic cells must then degrade or remodel basement membrane,

detach from the primary tumor mass and intravasate. Tumor cells must next survive the stress of vascular transportation and evade host defense mechanisms [12]. Attachment to their preferred site of metastasis may be either targeted, via tissue-specific microvessel cell adhesion molecules (CAMs) [14,15], or more general in nature simply allowing the tumor cells to reside in the target tissue long enough to respond to transendothelial soluble factors from the target organ (Sikes, unpublished data) [11]. Tumor cells then extravasate into the target organ parenchyma, where they proceed to proliferate in the new, supportive microenvironment as micrometastases. Finally, the micrometastases must induce angiogenesis to support growth of the new lesion [16].

Despite intensive research efforts very little is known about the specific mechanism(s) that contribute to the predominant pattern of PCa metastasis and establishment of bony lesions (Sikes, unpublished data). Various theories have been proposed, including venous drainage, lymphatic spread, and arterial emboli [5]. One of the oldest, for example, is based on anatomical juxtaposition of the veins draining into the lower vertebral column and pelvic girdle from the testes, penis and prostate gland called Batson's plexus [17,18]. Data indicate that lymphatic channels are only capable of transporting metastatic cells to regional nodes, and further dissemination is carried out through the vascular system [17,18].

Prior to reaching the circulation for transport to and subsequent re-colonization at a metastatic site, PCa cells must become motile and detach from the primary tumor and overcome the barrier of the extracellular matrix (ECM). Cell locomotion is a coordinated balance between adhesion and detachment of cells through CAMs that occurs simultaneously with tumor cell-induced remodeling of ECM [19-21]. Tumor cell adhesion may be modulated by cytokines and growth factors (GFs) that effect CAM expression and functional organization [22]. To date at least 50 CAMs have been identified and are divided into four major families based on protein structure: 1) the Ig superfamily, 2) the cadherin family, 3) integrins and 4) selectins [23]. Additionally, upregulation of matrix-metallo proteinases (MMPs) and other protease-receptor pairs, like protease-activated receptor 1 (PAR1), facilitate invasion through the basement membrane, providing necessary access to either the lymphatic or vascular circulations [24].

LNCaP and PC-3 (Table 1) models are the two principal culture-to-animal systems being used to delineate the specific mechanisms required for bone metastasis, and will be the focus of experimental data presented in this review, unless otherwise stated. Particularly, the LNCaP model of PCa progression [25-27] gives an opportunity to follow coordinated changes in integrin expression, usage, and behavior of PCa cells when exposed to different ECM substrata and stromally-derived soluble factors; and is unique in that all cell lines vary in metastatic potential but share a common genetic background. Previous phenotypic [27] and genotypic [28] characterizations of these cell lines also revealed their remarkable resemblance to the clinical progression of human prostate cancer. The goals of this review are to discuss the current data that point to tumor- and ECM-derived factors as major contributors of PCa metastasizing to bone, with specific attention to soluble and insoluble factors, CAMs and proteases that mediate PCa cell detachment from the primary tumor, migration and invasion to and through the ECM. At the end of each section, we will attempt to briefly integrate what the data demonstrates in light of key paracrine signaling mechanisms in both the tumor and ECM microenvironments, and explain how we believe these mechanisms may drive metastatic PCa progression. The various prostate cancer cell lines discussed in this review are briefly described in Table 1.

Table 1: Basic Properties of Prostate Cell Lines.

Cell Line	Origin	Androgen Responsiveness	Tumorigenicity
NbE1.4 [151-154]	Normal rat ventral prostate	Androgen sensitive	None
P69 [148,149]	Normal Human prostate	Androgen independent	Very poor
DU-145 [147]	Human brain metastasis	Androgen independent	High
LNCaP [145]	Human lymph node metastasis	Androgen sensitive	Low
C4-2* [113,144]	Human castrated mice	Androgen refractory	High with bone metastases
VCaP [150]	Human bone metastasis	Androgen sensitive	High
PC-3 [146]	Human bone metastasis	Androgen independent	High

*This cell line was derived from the original co-inoculation of LNCaP and bone stromal cells in mice. References for each cell line are as follows: LNCaP (Lymph Node Carcinoma of the Prostate), PC-3 (Prostate Cancer-3), DU-145 (Dura-145), and VCaP (Vertebral-Cancer of the Prostate).

The extracellular matrix and prostate cancer progression

The ECM or basement membrane of most epithelial sheets is primarily composed of laminin and collagen type IV (coll IV), as well as other collagen subtypes. In addition, the ECM also consists of many non-collagenous molecules such as bone sialoprotein (BSP), osteopontin (OPN), osteonectin, osteocalcin (OC), fibronectin, vitronectin (VN), and thrombospondin [29]. As PCa progresses, the expression of many of these components is up-regulated, down-regulated, or lost all-together. These differential patterns of expression aid the tumor in ECM transmigration and ultimately metastasis. For example, when prostate cells are transformed with the *neu* oncogene, collagen IV is overexpressed in PCa cell lines PC-3 and NbE1.4 [30,31]; while laminin alone is up-regulated in NbE1.4 [31] and the calcium-independent intercellular cell adhesion molecule-7 (ICAM-1) is down-regulated in PC-3 cells [30]. In the LNCaP progression model, BSP [32,33], OPN [34] and OC [35] are overexpressed in the more aggressive C4-2 cells. Multiple studies have also demonstrated changes in expression of ECM molecules in advanced PCa tumor samples, such as increased expression of BSP and Cadherin 11 [36,37], or decreased coll VII expression [38]. Xue *et al.*, found an interesting pattern of expression in Tenascin-C, a molecule involved in stromal-epithelial interactions, where low and moderate-grade tumors showed high levels of the glycoprotein that diminished in high-grade tumors [39]. In the same study, they also documented a dramatic loss of laminin expression indicating a break in the basement membrane adjacent to the tumor cells. These findings were recently corroborated by Brar and colleagues [40].

Cadherins and prostate cancer progression

Cadherins include a multigene family of cell surface adhesion glycoproteins, that provide homotypic interactions between cells and are used to maintain cell:cell associations or mediate cell migration [41]. Classical cadherins (E-, N-, and P-cadherin) possess a highly conserved and characteristic cytoplasmic domain that interacts with their functional partners, β- or γ- and α-catenin [42]. Loss of expression of either pair in this functional complex has been associated with an invasive phenotype due to reduced cell:cell adhesion [43-45]. E-cadherin is an epithelial-specific, calcium dependent CAM that functions to maintain epithelial sheet integrity [46]. As carcinomas become more aggressive with propensity to metastasize, E-cadherin is often lost [46], making it a good candidate to be a metastasis suppressor. It may also serve as a useful prognostic marker for PCa because it is lost with increasing tumor grade and stage [47,48], and down-regulated in PCa cell lines, PC-3, LNCaP and C4-2 [37,49,50]. The nature of the down-regulation is by both protein level and proteolysis resulting in loss of function. Conversely, the expression of N-cadherin in PC-3 cells [49] and advanced prostatic carcinomas [37] has been demonstrated to increase. This would be expected because N-cadherin expression in highly invasive breast cancers was found to replace E-cadherin in facilitating cell:cell contacts, and thought to mediate the interaction between mammary tumor and stromal cells [51]. It is therefore thought to contribute to metastasis via mammary stroma migration [52], and may function similarly in PCa.

In addition to classical cadherins, Bussemakers *et al.*, found other cadherins, including -4, -6, and cadherin-11 were also expressed in a number of PCa cell lines such as PC-3 [37]. In particular, they found an increased expression of cadherin-11 and one of its splice-variants, previously associated with dominant-negative regulation of cell adhesion [53], suggesting it plays a role in PCa progression [37]. Interestingly, they also showed no detectable mRNA or protein expression of cadherin-11 in the less aggressive LNCaP cells.

Role of integrins during progression

The expression of CAMs on cancer cells, as well as on endothelial cells, is not static, but dynamic and strictly controlled by mediators such as GFs, cytokines/chemokines, and the composition of the ECM [22,54-58]. Cell behavior decisions, such as decreasing cell-cell and cell-substrate attachment, and increasing cell motility are accompanied by changes in the expression and/or usage of adhesion receptors,especially those of the integrin family [59,60]. Integrins are themselves heterodimeric molecules, consisting of one α and one β subunit, with at least 20 different combinations already described, many of which differ in their extra- and intracellular binding specificities [61,62]. Integrin molecular structure, heterodimerization, and intra- and extracellular interactions with cytoplasmic regulatory proteins and ECM ligands provide tremendous potential for variation among cell types, well beyond that available through quantitative variation in integrin expression level alone [63].

Since integrins are intimately involved with cell adhesion and motility, experiments were performed to see if there were overt changes in the amount of cell surface integrins expressed in the LNCaP progression model and P69 cell lines. Compared to the cancer cell lines, P69 has much higher expression of integrin subunits (Sikes, unpublished data). These data correspond very well with the strong binding demonstrated in the adhesion assays and the low metastatic potential of this SV40-immortalized cell line (Sikes, unpublished data). Essentially, cells that adhere too well cannot move well. A direct comparison between the cell lines of the LNCaP lineage revealed very few changes in the absolute levels of integrin subunit expression. Only the α_2 and β_5 integrins increased

appreciably. While $\alpha_2\beta_1$ was shown to bind laminin (LN) and collagen [64], our previous work [63] indicates that this integrin pair is probably not utilized in the LNCaP progression model to bind LN. Integrin $\alpha_2\beta_1$ also mediates PC-3 cell adhesion to collagen I, which is a major component of the bone matrix [57]; and was found to mediate cell adhesion to collagen type II, III and IV as well [65].

Despite the modest changes in the absolute levels of integrin subunits, we demonstrated that LNCaP and C4-2 cells have switched the functional pairing of integrin heterodimers [63]. As described by Edlund *et al.*, LNCaP uses primarily $\alpha_6\beta_4$ and not β_1 pairs to bind LN, VN and OPN, while C4-2 uses a combination of $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins to bind these matrix components [63]. This switch in integrin heterodimer usage reflects a shift from a junctional integrin expression by LNCaP, $\alpha_6\beta_4$, to a motility associated pair of integrin heterodimers in C4-2 cells, $\alpha_3\beta_1$ and $\alpha_v\beta_3$. [63]. Additionally, the $\alpha_6\beta_1$ pair is up regulated in C4-2 cells and has been associated with both an increase in metastatic behavior and enhanced cell spreading in many prostate cancer cell lines [31,63]. In tissue, an increase in $\alpha_6\beta_1$ expression was associated with invasion of the seminal vesicles by prostate cancer [66]. Cooper *et al.*, demonstrated that the β_1 subunit is involved in PC-3 cell adhesion to fibronectin, a soluble ECM component of the bone microenvironment [67]. These data would tend to rule out a direct effect of this integrin in the adhesion of either LNCaP or C4-2 cells, but does not rule out a role for this pair in cell motility or invasion, especially considering that the role of β_1 in invasion and motility has been aptly demonstrated for endometrial cancer [68]. Direct evidence of this in LNCaP and C4-2 remains to be confirmed experimentally.

Refocusing on the LNCaP model, the more metastatic sublines were distinct in their use of $\alpha_v\beta_3$ and, when compared with parental cells, showed a shift in α_6 heterodimerization, a subunit critical not only for interaction with prostate basal lamina but also for interaction with the bone matrix [63]. The involvement of the $\alpha_v\beta_3$ pair is unusual in epithelial cells since it is usually expressed in lymphocytes and other migratory cell types [69,70]. It is clear, however, that C4-2 cells are using $\alpha_v\beta_3$ integrin heterodimers for both adhesion and migration on LN, OPN and VN, while LNCaP cells do not [63,71,72]. Interestingly, breast and lung cancer cell lines that were derived from bone marrow aspirates also expressed $\alpha_v\beta_3$, suggesting that it plays a role in overall metastasis of cancer cells to bone marrow [70]. PCa cell adhesion to and migration on components present in the bone matrix are also mediated, in part by $\alpha_v\beta_3$ [73]. For example, a number of studies have shown that both breast and PCa cells attach to bone-specific ECM components following transendothe-

lial migration, including vitronectin and osteopontin [14,22,29,74,75]. The $\alpha_v\beta_3$ expressed on PCa cells, is also a natural receptor for many of the previously mentioned non-collagenous ECM proteins [22,75-77]; and may give an indication as to why C4-2 cells, known to spontaneously relocate to bone [27], increase their use of this integrin pair as compared to $\alpha_6\beta_4$. It is also relevant to note here that compared to a number of other PCa cell lines, $\alpha_v\beta_3$ expression was recently determined to be the greatest in PC-3 cells [11]. Thus, the data suggest that $\alpha_v\beta_3$, in part, facilitates PCa metastasis to bone by mediating PCa cell adhesion to and migration on OPN and VN, two dominant proteins in the bone microenvironment [59].

The mechanism involved in coordinating the heterodimer usage between these cell lines has not yet been determined. Curiously, our FACS analysis of live cells would indicate that the levels of surface α_6 and β_4 have not changed between LNCaP and C4-2. Even-so, $\alpha_6\beta_4$ use declined in C4-2 cells, in conjunction with an increased use of $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_3\beta_1$ [63]. This was reiterated by the observation that the striking increase in the spreading of C4-2 cells on LN after treatment with stromal factors could be completely obliterated by the addition of function-blocking antibodies against α_6 or β_1, but not against α_2, α_3, β_4, or $\alpha_v\beta_3$ [63]. Since $\alpha_6\beta_4$ heterodimers participate in both the formation of hemidesmosomesas well as in the control of cell motility by unique properties of the β_4 integrin [78], it is possible that the β_4 subunit or $\alpha_6\beta_4$ heterodimer are actively participating in the motility and invasive behavior of PCa cells in a manner that is different from that used by LNCaP cells to attach to a substrate. The function of the β_4 integrin would then be determined in a context-dependent manner interpreting environmental cues. In primary prostate carcinomas and established PCa cell lines (DU-145), the α_6 integrin subunit maintains a persistent expression during PCa progression, and shifts in β subunit heterodimerization partners were observed from $\alpha_6\beta_4$ alone to also include $\alpha_6\beta_1$ pairs [38,79]. In other tumor cell types, these laminin-binding integrins ($\alpha_6\beta_4$ and $\alpha_6\beta_1$) have also been linked to acquisition of invasive behaviors [79,80]. The shift in α_6 usage concurs with previous studies, where $\alpha_6\beta_1$ and $\alpha_6\beta_4$ were both found in normal prostate cells, but β_4 subunit expression was lost in carcinomas [38,80,81]. Taken together, these data indicate that functional changes in surface proteins that are involved in invasion are likely to occur with or without major changes in levels of the protein expressed and that these changes are dependent on the epithelial-ECM-stromal interactions within the tumor and subsequent bone microenvironments (Sikes, unpublished data).

The data also indicate that human prostate cancer has altered integrin expression when disseminated to the bone. When cancer tissue was compared to hyperplastic or benign tissues, the alterations in integrin usage in cancer were found in laminin-binding integrin expression in particular [74,79,82,83]. For example, a number of studies showed decreases in the expression of the $\alpha_6\beta_4$ integrin pair [81], β_4 expression alone [84] loss of polarity [85]; as well as decreased α_2, α_4, and α_v expression [86] in more advanced PCa tissues as compared to non-invasive samples. Additionally, Murant *et al.*, found an increase in the β_1 subunit as PCa progresses while Zheng *et al.*, documented once more the trend of increased expression of the $\alpha_v\beta_3$ integrin pair in advanced carcinomas [74,87].

Multiple pathways resulting from extracellular and intracellular signals regulate invasion of a carcinoma cell. Indeed, cell migration results from the merging of signaling pathways that employ GFs and their receptors, adhesion receptors (integrins) and cytoskeletal elements [88]. In one investigation, Aprikian *et al.* demonstrated that $\alpha_v\beta_3$ was involved in bombesin, a neurotransmitter and a cancer growth factor, stimulation of PCa cell motility [89]. Neuroendocrine cells in PCa express and secrete bombesin-like peptides, suggesting that these peptides are involved in PCa progression [89]. Bombesin increased PC-3 cell invasion through matrigel, but did not alter its adhesion to ECM proteins including VN [89]. Additionally, bombesin treatment was found to cause β_1, β_3, and β_5 integrin subunits to coimmunoprecipitate with focal adhesion kinase (FAK) [89]. Functions of $\alpha_v\beta_3$ in PCa cells are mediated by FAK, which activates the phosphatidylinositol 3-kinase (PI-3 kinase)/Akt pathway [74,76]. The PI3K/Akt pathway may also be involved in androgen-independent growth of PCa [34,90-94]. Once activated by an upstream kinase such as FAK, this pathway facilitates cell survival and proliferation by increasing expression of the cell cycle regulator E2F, which mediates progression through the cell cycle; as well as prevents the pro-apoptotic activity of BAD [95,96]. FAK also activates NF-κB, which is known to regulate the transcription of anti-apoptotic proteins [96]. PC-3 cells adhered strongly to collagen type I, a major component of mineralized bone matrix, in the presence of TGF-β (10 ng/ml), a growth factor found in high levels in the bone matrix, and this interaction was mediated by integrins $\alpha_3\beta_1$ and $\alpha_2\beta_1$ [29,57,97,98]. Kiefer and Farach-Carson [29] demonstrated that PC-3 cell adhesion to collagen type I stimulated an increase in cyclin D1 expression followed by an increase in cell division. This implicates the activation of PI3K, map kinase (ERK1/2) and p70S6 kinase in the collagen-mediated effect on PC3 cells.

Changes in extracellular matrix proteases

The ECM is a barrier to a progressing cancer cell at both the primary and metastatic sites. To overcome the ECM barrier, cancer cells alter their production of specific proteases that degrade components of the ECM. Changes in several of these proteases have been associated with prostate cancer progression as described below.

Matrix-metalloproteinases (MMP) are a family of zinc-dependent endopeptidases with broad substrate specificities for a variety of ECM/BM components, such as collagen types I, II, III and IV, laminin and fibronectin [99]. As tumor cells grow and divide, they secrete MMPs that break down the stroma and basement membrane [11]. At the same time, there is down-regulation of tissue inhibitors of MMPs (TIMPs) that amplify the process [100]. In fact, some members of the MMP family of proteases may associate with cell membrane receptors able to drive an oriented degradation of ECM and display a disintegrin region that, by virtue of an RGD motif, play a role in cell-cell adhesion and cell migration (ADAM proteinases: A Disintegrin And Metallo proteinase) [101]. Previous studies have demonstrated the importance of MMPs associated with tumor and stroma as critical determinants for ECM deposition, remodeling and the establishment of PCa metastases in the bone [20,102,103]. Changes in expression of specific MMPs are reported to be associated with PCa progression. Lichtinghagen *et al.*, recently demonstrated that MMP-9 protein was significantly higher in cancerous prostate tissue compared to normal prostate tissue [104]. There was no significant difference in MMP-2 expression between cancerous and normal tissues; however, there was a significant difference in the ratios of MMP-2 and MMP-9 to the tissue inhibitor of metalloproteinases 1 (TIMP-1), with cancerous tissue having a higher ratio. Although MMP-2 protein level is not altered during prostate cancer progression, an earlier study with prostate cancer cell lines demonstrated that the expression of membrane-type 1(MT1)-MMP, which activates proMMP-2 and is expressed on the surface of invasive cells, is up-regulated in PC-3 and DU-145 cells [105]. Since the main component of the basement membrane (BM) is collagen type IV, a substrate for both MMP-2 and MMP-9, it is conceivable that the higher expression of MMP-9 and MT1-MMP and the higher ratios of MMP-2 and MMP-9 to TIMP-1 play important roles in the destruction of the BM necessary for invasion and metastasis.

Neutral endopeptidase (NEP)-24.11 (neprilysin) is another cell surface metallopeptidase that may be involved in prostate cancer progression [106]. NEP degrades a variety of bioactive peptides including endothelin, which has been implicated in the growth of hormone refractory prostate cancer. Usmani *et al.*, reported that NEP expression was down-regulated in

advanced prostate cancer cell lines PC-3 and DU145 compared to LNCaP and normal immortalized prostate epithelial cells [106]. Also, NEP expression was down-regulated in cells derived from malignant tissues taken from radical prostatectomies compared to those cells derived from benign prostatic hyperplasia (BPH). We speculate that the down-regulation of NEP may contribute to an increase in bioactive peptides required for prostate cancer growth and metastasis

Cathepsins, a family of cysteine proteases capable of degrading several ECM components including coll IV, fibronectin and laminin [107-109]; are up-regulated during PCa progression. One study showed that the expression of Cathepsins (Cath) B and S was higher in prostate cancer tissue compared to BPH and normal prostate tissues; and, they were frequently co-expressed early in the development of prostate cancer [110]. Sinha *et al.*, reported that CathB activity was elevated in prostate cancer tissue samples compared to BPH and normal tissue samples [111]. This study also showed, by biochemical and immunogold electron microscopic analysis, the association of CathB with the plasma membrane as well as in lysosomes [111]. Brubaker *et al.* recently reported that CathK was expressed in prostate cancer tissues and not in normal prostate tissues [112]. The expression was variable in primary prostate cancer samples and soft-tissue metastases, but was consistently elevated in bone metastases. Surprisingly, CathK expression in PC-3 cell line, which was derived from a bone metastasis, was low compared to DU145 (derived from a brain metastasis) and LNCaP (derived from a lymph node metastasis). Furthermore, more advanced sublines, C4 and C4-2, of the LNCaP progression model, demonstrated decreasing expression of CathK. Note here, the subline C4-2 has a strong tendency to metastasize to bone in murine hosts [113]. Together this information demonstrates that Cathepsins contribute to prostate cancer metastasis and can be up-regulated by the bone microenvironment, a preferred site of metastasis.

The activation of protease-activated receptor 1 (PAR1; thrombin receptor) by thrombin may stimulate prostate cancer cells to secrete MMPs [11,24]. Chay *et al.*, showed that PAR1 expression was up-regulated in PCa compared with normal prostate tissue. This overexpression was very pronounced in bone-derived PCa cell lines (VCaP and PC3) compared with soft tissue PCa cell lines (DUCaP, DU145, and LNCaP), suggesting the PAR1, like CathK, is up-regulated by stromal factors in the bone marrow [11,24]. Currently, little is known about the role of PAR1 in prostate cancer progression and studies are underway to determine the effect of PAR1 stimulation on MMP-9 expression in prostate cancer cells. The exact function of PAR1 expression and thrombin in PCa metastasis has also not been delineated; however, these data suggest that

PAR1 enhanced expression on bone-derived PCa cells may be important in targeting these cells to the bone [11].

The urokinase-like plasminogen activator receptor (u-PAR) is a membrane-associated serine protease receptor for urokinase or the urokinase plasminogen activator (u-PA). U-PAR is a three-domain molecule. Each domain is numbered 1 to 3 from the amino-terminus to the carboxy-terminus. Domain 1 is the only domain involved in (pro)-u-PA binding, domain 3 also participates in providing the u-PA binding site. The receptor (u-PAR/CD87) focuses the enzymatic activity of u-PA and allows activation of plasminogen (PG) at the cell surface, which in turn, is bound to the cell membrane by α-enolase receptors [114] or to other cell surface proteins endowed with C-terminal lysyl residues, and to plasmin (PL) [101]. PL, a serine proteinase, similar to trypsin, acts almost exclusively when associated with the plasma membrane, because only in that location is it resistant to its inhibitor, α 2-antiplasmin [101]. Plasmin is the main protease involved in (pro)-u-PA activation, which gives origin to the initiation of the classical protease cascade (plasmin, interstitial MMPs, MT1-MMP, Gelatinase A) leading to ECM degradation.

Three extracellular protein ligands involved in ECM degradation and cell adhesion have been identified for u-PAR, namely u-PA, vitronectin (VN), and kininogen. VN and the two-chain form of high molecular weight kininogen (HMWK) share overlapping and mutually exclusive binding sites for u-PAR domains 2 and 3. HMWK-bound kallikrein (kall) may activate the conversion of u-PAR-bound (pro)-u-PA to u-PA, thus providing an alternate pathway to the one triggered by plasmin to (pro)-u-PA activation. Many indications suggest that the u-PA/u-PAR system, together with specific inhibitors of plasminogen activators (PAIs), is an organizer of cell-ECM contacts and covers the full range of activities required to promote and disrupt cell attachment sites [115]. PC-3 and C4-2 secrete more u-PA than LNCaP cells [116,117], which is most notably involved in the regulation of ECM-laminin degradation, thereby allowing for PC-3 and C4-2 cells to behave more aggressively. Indeed, in a later study conducted by Festuccia *et al.*, the malignant phenotype of PCa cells (LNCaP, C4-2, PC-3 and DU-145) was correlated with both u-PA and u-PAR expression [118]. They found that differential production of u-PA corresponded with the ability of the more aggressive lines to bind and activate plasminogen; thus providing direct support that u-PA secretion and the levels of u-PA- u-PAR complexes characterize the invasive phenotype of these cells [118]. These blocking antibody experiments also provided evidence that this pattern of expression correlates with stage and grade in prostatic carcinomas, making u-PA or plasmin candidate target molecules for metastasis-inhibiting therapeutics.

Several other molecules interact 'constitutively' with u-PAR domains 2 and/or 3, thereby functioning as 'coreceptors'. These molecules include the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2MR/LRP), the mannose-6-phosphate receptor (Man6PR)/ IGFII-receptor, gp130, u-PAR-associated protein (u-PARAP) and integrins [101]. Integrin family members including β_1, β_2 and β_3, may interact with u-PAR domains 2 and 3 as u-PAR co-receptors, which leads to an enfeeblement of integrin-ECM interactions [119]. If the cell expresses low or no caveolin, u-PAR-integrin complexes remain loose, floating on unspecialized areas of the cell membrane, integrin function will be impaired, and the cell-ECM interaction will rely solely on adhesive interactions mediated by u-PAR-VN [120]. This seems to be the case for leukocytes and transformed cells, where the motile properties of the cell must prevail on the cell-ECM moorings. On the contrary, if the cells express high levels of caveolin, u-PAR, as with many other GPI-anchored proteins, form clusters on caveolin-rich membrane rafts, together with the loose integrins. In this case, the complex "signalosome" of caveolae, rich in kinases of the src family, transduce signals leading to integrin overexpression, which reinforces cell-ECM interactions. This situation has been recognized in macrophages and metastatic tumor cells and results in enhanced adhesion and migration on ECM components [101]. The end result of the involvement of such a large range of signaling molecules is the activation of several groups of intracellular kinases such as Src, Src-like protein kinases (Hck and Fgf) and again FAK with convergence on the extracellular regulated kinase (ERK)1/2 pathway [121].

Summary and conclusions

The expression of CAMs on tumor cells is not static, but dynamic, and is regulated strictly by extracellular cues like soluble GFs, cytokines, and the insoluble proteins composing the ECM [54-58,122,123]. Although a number of integrin variations during PCa cell progression have been described [38,74,79-81,124-126], neither modulation of these variations by external factors nor integrin heterodimer usage regulation is well understood [63]. "Inside-out" regulation of integrin heterodimer activity and subunit partner choices are thought to depend on unique cytoplasmic regulatory protein repertoires that differ among host cell types [127-130]. Likewise, "outside-in" regulation by integrins, in response to extracellular cues, has revealed shifts in integrin gene expression as well as changing integrin associations with numerous signaling molecules, including protein tyrosine kinases (FAK and pp60src), serine kinases (protein kinase C, extracellular signal-regulated kinase, c-Jun-NH$_2$-terminal kinase, and integrin-linked kinase), and lipid intermediates (PI3K and phosphatidylinositol 4,5-kinase) [62,131-133]. Hence, integrin activity within a given cell is tightly coor-

dinated with its cell cycle, gene expression profiles, differentiation, and cell survival [61]. In our studies, few shifts in integrin expression were found to accompany PCa disease progression, while integrin heterodimer usage, changed significantly [63]. Changes in integrin expression or the functional reassortment of the heterodimers as a tumor progresses has been studied in both the PC-3 and LNCaP PCa cell line models but the mechanism has not been elucidated.

The final component to this story includes the protease-receptor complexes, which are also molecular organizers of cell-to-ECM interactions. They coordinate both adhesive and degradative activities necessary to facilitate metastatic progression [101]. Because traversing the ECM is a critical step in the invasive process, it is imperative that the mechanisms driving the conversion of stationary tumor cells to ones with the capacity to migrate be elucidated. Various cell-associated serine proteases and their respective receptors have been shown to up-regulate u-PAR, giving the u-PA/u-PAR/PAI-1 system prognostic significance in several tumor types [101], including PCa. As information about which u-PAR domains and u-PA sequences specifically mediate malignant invasion via proteolysis and adhesion increases, the likelihood of improved rational drug development to control the factors of the fibrinolytic system should also increase.

Integrin regulation of prostate epithelial proliferation is likely to involve interactions between these CAMs and GF receptors [63]. Such interactions are used by cells to interpret positive and negative GF and cytokine signals from surrounding stromal cells [134,135], via common signaling cascade components (e.g., small GTPases), also important for integrin signaling and activation. Preferential associations between the GF receptors and the changing integrin heterodimers could have dramatic consequences on the responses of a cell to environmental cues [63]. In LNCaP cells, $\alpha_6\beta_4$, is very important for attachment and would tend to restrict cell migration; while $\alpha_6\beta_1$ and $\alpha_3\beta_1$, both of which are involved in the formation of dynamic focal contacts cycled during migration, are important for cell locomotion [79]. In C4-2 cells, spreading in response to stromal factors appears to be mediated through $\alpha_6\beta_1$ which, along with $\alpha_v\beta_3$ are responsible for migration [63]. Similarly, we found the $\alpha_6\beta_1$ heterodimer to be more involved in cell spreading than static cell attachment in either *neu*-transformed cells or the LNCaP progression model [31,63]. The ability of $\alpha_3\beta_1$ to alter laminin chains and overall basement membrane architecture [136,137] is particularly suggestive, given that proteolytic cleavage of laminin can drive cells from static adhesion to active migration [138,139]. Although β_1 was shown not to be responsible for PC-3 cell invasion, it may still play a role in C4-2 cell migration/invasion [140].

Table 2: Summary of Molecular Changes Associated with Prostate Cancer Metastasis

Cell line	ECM	Proteases	Integrins
VcaP		↑PAR [24]	↑αVβ3 [11]
PC-3→PC-3(Neu-T)	↑CollIV [30]	↑PAR [24]	↑αVβ3 [11]
PC-3M	↓ICAM-1 [30]	↑MT1-MMP [105]	
	↑N-Cadherin [49]	↓NEP [156]	↑α2β1 [97]
	↓E-Cadherin [49]	↑Cathepsin D [157,158]	
	↑Cadherin 11 [37]	↑uPA [116]	
DU-145			
		↑MT1-MMP [105]	
		↑MMP-9 [159]	
		↓NEP [156]	
		uPA [116]	
LNCaP→C4-2	↑OSC [32]	↑uPA [116]	↑α3β1 (usage) [63]
	↑OPN [27]	↑PSA [34]	↑α1 (usage) [63]
	↑BSP [32]	Cathepsin D (LNCaP) [157,158]	↑αVβ3 (usage) [63]
	E-Cadherin [155]		↓α6β4 [63]
			↑α2 [63]
			↑α6β1 [63]
NbE1.4 (Neu-T)			
	↑Laminin [31]		
	↑CollIV [31]		↑α6β1 [31]

Tissues

Cell line	ECM	Proteases	Integrins
Benign→Malignant	↑BSP [160]	↑Cathepsin B^A [110,111]	↑β1 subunit [87]
	↑Cadherin 11 [37]	↑Cathepsin S [110]	↓α6β4 [81]
	↑N-Cadherin [49]	↑MMP9 [104]	↑αVβ3
	↓E-Cadherin [87]	↑Cathepsin K "bone" [112]	↓β4 [84]
	↓Laminin [40]	↑HK2 [161]	↓β4 (loss polarity) [85]
	↑Tenascin C [39]	↑MMP2 [162]	↓α2 [86]
	↓Collagen VII [81]	↑MMP7 [163]	↓α4 [86]
		↓TIMP1 [163]	↓αV [86]
		↑Cathepsin D [164]	
		↑MMP26 165	

VCaP, PC-3, and DU-145 are advanced prostate cancer cell lines and were compared to the less advanced LNCaP cell line. The information under cell lines reflects this comparison. Please note the following: "bone" refers to observations specific for the bone-derived cell lines or malignant tissue from the bone, ↑ refers to an increase expression and ↓ refers to a decrease expression, OSC refers to osteocalcin, OPN refers osteopontin, superscript A refers to increased activation, MMP is matrix-metalloproteinase and LNCaP→C4-2 refers to LNCaP progression to androgen-independent C4-2 subline.

Most notably, the majority of these functions are directed by $\alpha_6\beta_4$ in LNCaP, suggesting that a functional reassortment of integrins occurs coincident with the acquisition of additional metastatic traits by C4-2 [140]. Not frequently found in epithelial cells, $\alpha_v\beta_3$ is common to a number of bone metastases, including those of prostate and breast carcinomas [74,126,141]. Two possible consequences of $\alpha_v\beta_3$ heterodimer usage in the metastatic LNCaP sublines are (*a*) preferential relocation to the bone and (*b*) increased cell survival/suppressed cell death [63]. Outside of the role of $\alpha_v\beta_3$ in binding metastatic cells to the bone matrix, this integrin heterodimer is also a good candidate regulator of cell survival in the absence of cell adhesion [63], and thus will remain in the focus of targeted anti-invasive therapies. Although loss of appropri-

ate adhesion is normally a cue for apoptosis, human breast cancer cells are able to use $\alpha_v\beta_3$ to inhibit p53 activity and suppress the bax death pathway [142]. Accordingly, $\alpha_v\beta_3$ has been shown to regulate cell proliferation in prostate epithelia [143]. The various patterns of differential expression of ECM molecules, proteases and integrins discussed in this review have been summarized in Table 1.

One of the most notable trends in the area of PCa metastasis is the move towards more complicated *in vitro* and *in vivo* systems. This has become necessary since it is now apparent that studying the individual components, prostate or bone cells, or other components of their microenvironments alone, is clearly deficient. These cells alter their gene expression and migratory behavior in

response to co-culture or cross-feeding (personal communication Dr. Farach-Carson). Furthermore, the only way to effectively resolve the role of tumor versus ECM factors in the migration and downstream establishment of boney metastases will be to examine the behavior of these various cells types in structured mutlicellular assays. Candidate target proteins can be down regulated using antibodies, ribozymes or RNAi. Conversely critical proteins can be supplemented to systems of reduced complexity to test for the biological response or re-expressed in tumor cells to study the effects of their expression. The individual cells can be followed using fluorescent tags to visualize cell interactions. Once established, these models will allow for the functional analysis of ECM proteins, proteases and integrins involved in the dynamic journey of PCa cells from their site of origin to their interaction with factors and cells from the bone milieu that include ECM, stroma, osteoblasts and osteoclasts, to ultimately colonize the bone.

Acknowledgments

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