Chapter 6

Plasminogen Activator System — Diagnostic, Prognostic and Therapeutic Implications in Breast Cancer

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Additional information is available at the end of the chapter

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1. Introduction

As tumor metastasis to distant organs (lungs, liver, brain, bone) continues to be the leading cause of cancer associated morbidity and mortality, including breast cancer patients, therapies targeting genes involved in the metastatic cascade are a potentially effective strategy for blocking breast cancer progression and improving survival [1]. Previous 'one size fits all' cancer therapies, which have been used to treat a wide variety of cancers, are inefficient and often cause much unnecessary treatment-related toxicity. Thus, there is a huge unmet need in the research and medical community towards the characterization of cancers into more specific subcategories, which can then be used for prognosis and identifying potential therapies. However, this process requires the use of specific biomarkers to act as signatures for the different subcategories [2,3]. In breast cancer, the most commonly used biomarkers are the estrogen receptor (ER), the progesterone receptor (PR), and the epidermal growth factor 2 (HER2) oncogene [4]. More recently, the plasminogen activator (PA) system and its associated genes are being used as biomarkers to identify potential aggressive cancers, including in breast cancer. The urokinase-type plasminogen activator (uPA) and its inhibitor, the plasminogen activator inhibitor 1 (PAI-1), are proteins of the PA system which are distinguished among cancer biomarkers as being the first to attain level-of-evidence 1 (LOE-1). Thus, assessment of uPA and PAI-1 levels by ELISA assay has been a recommendation of the American Society of Clinical Oncology (ASCO) for assessment of the risk of reoccurrence in breast cancer patients since 2007 [5]. Elevated expression of uPA and its receptor (uPAR) are correlated with poor prognosis and are associated with advanced cancers, including occurrence of metastasis [6]. uPAR is unique as it is rarely expressed in normal quiescent tissue whereas its expression is uniformly high in several tumor tissues, identifying it as a good indicator of malignancy [7].

These characteristics and many more make the PA system an excellent biomarker for breast cancer diagnosis, and a promising target for future breast cancer therapies.

In this chapter, we will discuss the current state of knowledge and ongoing efforts to establish uPA-uPAR system as a diagnostic, prognostic and therapeutic target in breast cancer.

2. Molecular characterization of breast cancer

The wide variety of breast cancer-targeting therapies which exists is due in large part to the diversity in the manifestations of breast cancer. When characterizing these cancers into subtypes, in order to identify patterns, morphology remains the cornerstone for diagnosis [4]. However, molecular classification of breast cancers is being used more and more as an additional tool for prognosis and prediction of disease progression. Prognostic factors identify the severity of the disease, forecasting the outcome of the cancer in an untreated individual. Predictive factors are used to identify treatment options, given the characteristics of the cancer, and predict how beneficial a given treatment might be [8]. The most commonly used biomarkers for molecular classification of breast cancer are ER, PR, and HER 2, levels of expression of which are routinely determined by immunohistochemistry [4]. In addition to these, the nuclear protein Ki-67 is a good indicator of cell proliferation; higher levels of Ki-67 expression are associated with poor prognosis and identifies a point at which a patient is at an increased risk of developing distant metastases [9]. In order to establish a stronger prognostic test which takes into account breast cancer cell proliferation, the percentage of Ki-67-positive tumor cells has been combined with the HER2, ER, and PR scores to form the "IHC4". This prognostic test is powerful when used for ER-positive breast cancers [10]. In addition to immunohistochemical studies identifying key biomarker proteins, newer assays have been developed which use expression levels of mRNA to characterize breast cancers into different subsets [4,11-15].

3. Skeletal metastasis in breast cancer

Metastasis accounts for 90% of deaths in cancer patients [16]. In breast cancer specifically, 70% of patients dying of the disease show presence of bone metastases in their post mortem examination [17]. Cancer metastasis is the spread of cancerous cells to distant tissues, where the cells then go on to form colonies independent of the original source. The original source could be the primary tumor, or the circulating tumor cells could have originated from another metastatic tumor [18]. The process of metastasis is not a spontaneous event, but rather a concerted evolution, in which one cell or population of cells undergoes a series of alteration or mutations which render the cells their invasive and metastatic phenotype [19]. Breast cancer metastasis to the skeleton is a non-random metastatic process; the location of distant metastasis is not based on vasculature or blood circulation. Rather, it is known that certain tumors have an increased 'preference' towards metastasis in certain organs as first describe in the "seed and soil hypothesis" by Paget in 1889 [20]. In addition to breast cancer, cancers of the prostate,

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lungs, kidney, liver, and thyroid, all show predilections towards skeletal metastasis [1,21]. Thus, there is a continuous search to identify genes and proteins which are involved in initiation and progression of skeletal metastasis in breast cancer and which can be targeted to develop innovative therapies. Bisphosphonates are analogs of pyrophosphate, with a carbon atom replacing the central oxygen atom of the pyrophosphate molecule [22]. Bisphosphonates are rapidly deposited on the bone surface, where they are subsequently ingested by osteoclasts as the cells degrade the bone matrix. Once inside the osteoclast, they interfere with the resorption process by inducing a toxic apoptotic effect. Bisphosphonates can also inhibit osteoclast differentiation and maturation [22]. Due to these effects on bone remodeling they are routinely used in patient with osteoporosis. Bisphosphonates have also been shown to be effective in reducing the incidence and number of skeletal metastases in women with breast cancer who were seen as at-risk of developing distant metastases [23]. Phase II clinical trials have shown that the use of bisphosphonate therapy in conjunction with standard anti-cancer therapy is more effective in reducing the number and persistence of disseminated tumor cells than standard therapy alone [24]. There is also evidence which points to antiangiogenic activity of zoledronic acid, a commonly used bisphosphonate, supporting the rationale for its use in breast cancer therapy [25].

Another drug which targets osteoclast activity is Denosumab, an inhibitor of the receptor activator for nuclear factor kappa-b ligand (RANKL). RANKL is a key regulator of bone resorption which is secreted by osteoblasts and binds to the receptor activator for nuclear factor kappa-b (RANK) on osteoclast progenitor cells, thereby stimulating osteoclast activation and maturation [26]. Osteoblasts also secrete osteoprotogerin (OPG), which can bind to RANKL, acting as a soluble decoy receptor and preventing RANKL-induced osteoclast activation. Thus, osteoblasts have the ability to regulate the rate of bone resorption through the control of osteoclast activity [27]. RANKL levels have been found to be elevated in breast cancer cells, which results in excessive bone resorption [1]. It has also been shown that RANKL promotes the migration of RANK-expressing tumor cells to bone [28]. Denosumab is a fully humanized anti-RANKL monoclonal antibody, acting like OPG to block RANKL binding to RANK and thus preventing osteoclast activation and maturation [29]. Denosumab was originally developed as a treatment against osteoporosis in postmenopausal women, although it is now approved to treat skeletal related events in cancer patients as well [30]. Integrin $\alpha v\beta 3$ is a cell surface receptor found on osteoclasts which stimulates intracellular signaling of the c-Src cascade [31]. Preclinical studies have demonstrated that $\alpha v\beta 3$ integrin-inhibiting drugs can successfully blocked tumor growth and osteolysis [32,33]. Members of the integrin family including $\alpha v\beta 3$, are significant due to their interaction with the uPA-uPAR system.

4. Proteases and breast cancer

Cancer mortality is usually a result of the metastatic spread of the cancer to distant vital organs, as opposed to growth of the original tumor [34]. As such, it is crucial to understand the progression from the localized to an invasive cancer, and eventually a metastatic cancer. Along with growth factors and cytokines, proteases play a major role in this progression, causing the

degradation of the basement membrane and surrounding extracellular matrix. Proteases play a crucial role in this first step, as they digest the basal lamina components, and allow for cell movement through the extracellular matrix (ECM) [34]. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases whose primary role is the degradation of ECM proteins, dissolving connective tissue [35]. There are a total of 28 identified MMPs, of which 14 have been implicated in breast cancer development and progression [36]. MMPs are synthesized by the tumor itself as well as the surrounding peritumoral stromal cells [37]. In the area surrounding a tumor, the major source of MMP activity is the stromal cells, with the tumor cells likely stimulating production of MMPs via the local fibroblasts [38]. In order for the cancer to move beyond its original location and invade into a nearby duct, MMP activity must break down the basement membrane and stromal matrix, facilitating ECM remodeling [34].

The PA system in general and uPAR in particular play a significant due to its ability to localize the proteolytic effects of uPA which can activate latent growth factors and proteases to effect angiogenesis, matrix degradation, adhesion, activation intracellular signalling pathways, tumor cell invasion and metastasis depicted in Figure 1.

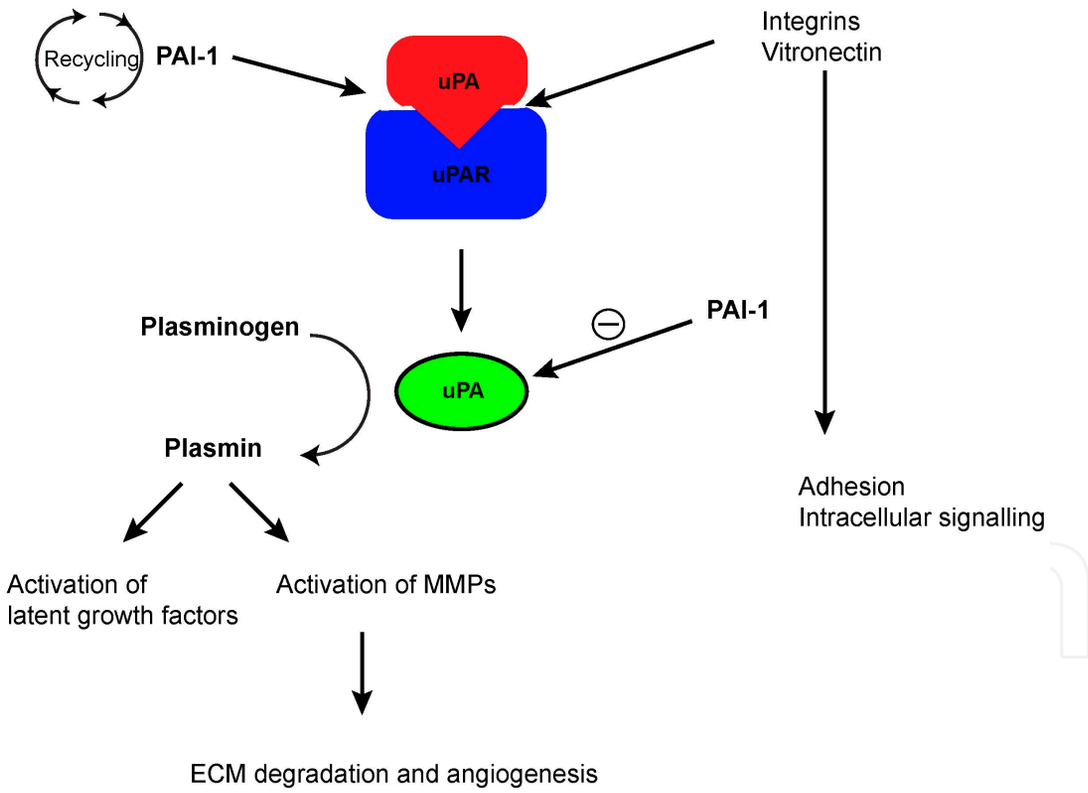

Figure 1. Central role of uPA and uPAR in tumor progression. uPA is localized to the tumor cells via its binding to domain 1 of uPAR. uPA can activate inactive zymogen plasminogen to plasmin, which can activate matrix metalloproteases (MMPs) and activate or release growth factors. Via its domains 2 and 3, uPAR can interact with integrins ($\alpha v\beta 3$, $\alpha v\beta 5$) and vitronectin. PAI-1 binding to the uPA-uPAR complex inhibits the activation of plasminogen by uPA, and promoted internalization of the uPA-uPAR-PAI-1 complex and recycling of uPAR back to the cell surface. Collectively, the uPA/uPAR system plays a central role in matrix degradation, angiogenesis, adhesion, intracellular signalling, tumor invasion and metastasis.

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5. Plasminogen Activator (PA) system

The plasminogen activator (PA) system is a key regulator of the tumor microenvironment, and is heavily implicated in the metastatic process in breast and other common cancers. It is involved in tumor recruitment of inflammatory cells, tumor cell growth and survival, angiogenesis, and tumor invasion and migration [39,40]. The PA system of enzymes comprises two plasminogen activators, tissue type plasminogen activator (tPA) which converts plasminogen to plasmin during clot lysis, and uPA which is used therapeutically as a fibrinolytic agent. tPA is present in normal and some malignant tissues, whereas uPA is more commonly associated with malignancies and plays a major role in pericellular proteolysis during cell migration and tissue remodelling (Figure 1) [41]. Within the PA system three key peptide members: uPA, uPAR and PAI-1 and 2 have now emerged as a viable and effective diagnostic, prognostic and therapeutic target in breast cancer patients [6]. uPA and uPAR expression have been shown to enhance tumor growth and metastasis [42,43]. Expression of uPA and uPAR is also correlated with poor prognosis, being associated with late stage disease, including metastasis [6]. This section will examine the members of the PA system, discussing their structures and functions, and will describe the important role this system plays in the progression of breast cancer.

5.1. Urokinase-type Plasminogen Activator (uPA) and plasmin

uPA is a serine protease expressed as a single chain zymogen, pro-uPA, which undergoes cleavage to form two-chain high molecular weight uPA (HMW uPA) [44]. After an additional proteolytic step, HMW-uPA is converted into an amino terminal fragment (ATF) containing the receptor-binding growth factor domain (GFD), and a proteolytically-active low molecular weight uPA (LMW-uPA) which retains its plasminogen activator (PA) function [45]. In previous studies, we identified the ATF of uPA as a selective mitogen for cells of the osteoblast phenotype [46-49]. uPA is composed of three domains: a kringle domain, a growth factor-like domain, and a serine protease domain [50]. The serine protease domain of uPA shows high specificity for its substrate, the inactive zymogen plasminogen, which it cleaves to form the activated protease plasmin; plasmin is responsible for the breakdown of various component of the ECM, exerting uPA's pro-invasive and pro-metastatic effects [51,52]. Plasmin is also a serine protease, and catalyzes the process of fibrinolysis, in which fibrin and other components of the ECM are degraded to allow for cell invasion, migration, and dissemination [52]. Plasmin promotes further tumor cell invasion through the conversion of pro-MMPs to enzymatically active MMPs. Plasmin can also promote tumor cell proliferation by activating latent growth factors. Thus, plasmin can also activate ECM degradation both directly and indirectly [53]. Interestingly, plasmin promotes a positive feedback loop in the ECM degradation process, as plasmin also cleaves pro-uPA to create HMW-uPA [52]. uPA synthesis and/or release can be induced by a variety of cytokines and growth factors, including EGF, VEGF, and TNF-α [54,55].

There is speculation regarding which enzyme is responsible for the cleaved activation of pro-uPA into uPA. It is hypothesized that plasmin may be the activator, however, this theory

results in ambiguity concerning whether uPA or plasmin is first activated and how [56]. Other enzymes, such as kallikreins, cathepsins, and matriprase, have been shown to be capable of cleaving single chain uPA (scuPA) *in vitro* and are speculated as potential 'first activators' [57]. Interestingly, *in vitro* experiments have shown that binding of pro-uPA to uPAR allows for activation of plasminogen into plasmin, despite pro-uPA not having been converted into it active form. It is thus believed that binding of pro-uPA to uPAR causes a conformational change that confers protease abilities to the single-chain molecule [58]. This is not entirely surprising, as a known role of uPAR is increasing the catalytic efficiency of uPA; in vivo, binding of uPA to uPAR greatly increases the efficiency of plasminogen conversion by as much as 50-fold [59].

Elevated expression levels of uPA in tumor tissue as compared with normal tissue have long been noted [60-62]. In both primary and metastatic tumors, uPA is localized to the invading front, which supports the theory that uPA plays an important role in tumor cell invasion and migration [63]. In breast cancer, increased levels of uPA are correlated with poor relapse-free and overall survival [64]. Increased expression of uPA is seen in patients several common cancers (breast, prostate, lung, colon, thyroid, glioma) where it promotes metastasis and indicates poor prognosis [65-70].

5.2. Plasminogen Activator Inhibitors (PAI)

The effects of uPA are neutralized by plasminogen activator inhibitors 1 and 2 (PAI-1 and 2), produced by stromal cells surrounding the tumor cells. PAI-1 and PAI-2 are involved in the tight control of proteolysis, causing the uPA-uPAR complex to be internalized [71]. Increased PAI-1 expression is associated with higher metastasis whereas PAI-2 has a protective role [72]. PAI-1 binding maintains the active conformation of the uPA-uPAR-vitronectin (VN) complex, interferes with cell matrix interactions, and acts as a detachment factor to promote tumor metastasis [73]. The uPA-PAI-1-uPAR complex is internalized via clathrin-mediated endocytosis, with help from the very low-density lipoprotein receptor (VLDLR) related protein LRP. Inside the cell, the uPA-PAI-1 complex dissociated from uPAR, and is trafficked to the lysosome for degradation. The unbound uPAR is then recycled to the cell surface [71,74]. Interest in PAI-1 as a target in malignancy was revealed in studies where an anti-PAI-1 antibody showed anti-invasive effects on melanoma and fibrosarcoma cells [75]. High-throughput screening led to the identification of small molecule inhibitors of PAI-1 with anti-angiogenic and polyp-formation inhibition activities, thereby identifying PAI-1 as a viable novel target for cancer [76,77].

5.3. uPA Receptor (uPAR)

The role of uPAR within the PA system goes beyond localizing the proteolytic activity of uPA. Rather, uPAR itself plays an important role in tumor progression, interacting with many key signaling molecules, a surprising discovery as uPAR is devoid of a transmembrane domain. Rather, uPAR is a three-domain protein covalently linked to the outer layer of the cell membrane by a glysocylphosphatidylinositol (GPI) anchor [6]. uPAR is important in localizing uPA to the cell surface, which is necessary for uPA's activation of

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plasminogen to plasmin [59]. All three domains (D1, D2, D3) are involved in the binding of uPA to uPAR, however only domains D2 and D3 are thought to play a role in uPAR's interactions with other cell surface proteins [6]. uPAR alters cell adhesion and signaling through the interaction with various cell surface proteins, such as integrins (including $\alpha v\beta 3$, $\alpha v\beta$, $\alpha 5\beta 1$, and $\alpha 3\beta 1$), G-protein coupled receptors (GPCR), VLDLR, and receptor tyrosine kinases (including epithelial growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR)) [7,78-80]. It is hypothesized that uPAR is part of a larger complex of signaling molecules, called a 'signalosome', which uses signaling effectors such as Src, Akt, and focal adhesion kinase (FAK) [81]. Many of these signaling effectors have been implicated in breast cancer progression, including Src, integrins/FAK, Ras/ERK, and Akt as depicted in Figure 2 [82]. As discussed in a later section, these effectors have become important drug targets for the inhibition of uPA/uPAR-induced breast cancer progression. uPAR is rarely expressed in physiologically normal tissue, although its expression can be up regulated during some pathological processes, such as wound healing or inflammatory response to infection [83,84]. It is involved in normal hemostasis, as plasmin plays an important role in fibrin clot lysis. Under those circumstances, plasmin proteolyzes ECM components either directly or through the activation of MMPs [85,86]. Importantly, uPAR is highly expressed in cancers, and can be expressed by the tumor cells themselves, as well as by tumor-associated cells such as stromal cells, endothelial cells, and infiltrating inflammatory cells [56]. uPAR-expressing tumors generally fall into two categories: those in which both tumor cells and tumor-associated cells express uPA and uPAR, and those in which only the tumor-associated cells express uPAR [56].

uPA and uPAR are not expressed homogeneously throughout the tumor, but instead are generally associated with the interface of tumor tissue-benign tissue or tumor and vascular tissue [87]. uPAR is generally expressed on the migrating or invading edge of cancer cells, restricting the region of proteolytic activity and providing directionality. Thus, a path is created through the ECM, in the direction of movement. A chemical gradient is also created for the invading cancer cells to follow, as chemotactic ECM fragments and latent growth factors are released in the path of ECM destruction [88]. The PA system is responsible for not only the migration of tumor cells, but is also implicated in the migration of tumor-associated macrophages. Binding of uPA to uPAR has different effects depending on the state of maturation of the monocytic cells. uPA-uPAR binding stimulates migration in less mature, more monocyte-like cells; this is would induce the cells to follow the uPA gradient towards the tumor site. On more mature, more macrophage-like cells, uPA-uPAR binding instead induces adhering; thus, a macrophage which arrives at the tumor site will remain [89,90]. uPA has also been implicated in angiogenesis, initially observed in models of corneal vascularization [91]. uPA proteolytic activity is required for endothelial cell migration, one of the earliest steps in angiogenesis, and is also required for the earliest stages in tube formation [92,93]. Thus, the PA system plays an important role in the progression of breast cancer, promoting proliferation through angiogenesis, and enabling metastasis through the induction of tumor cell invasion and migration.

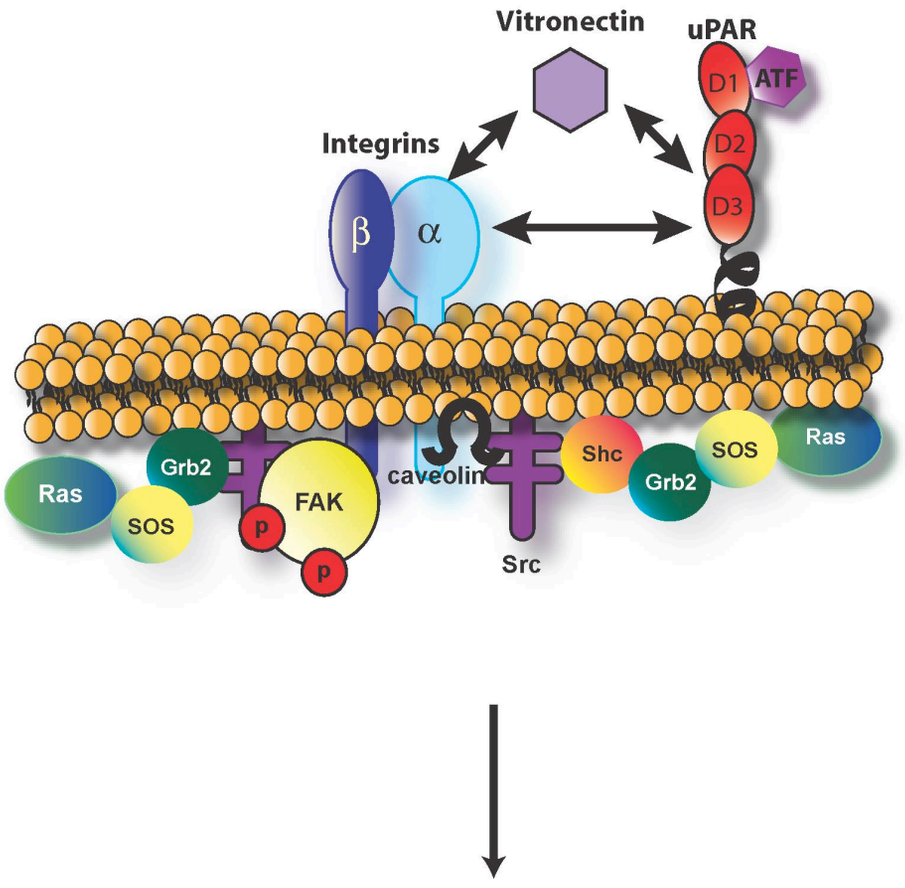

Figure 2. Schematic diagram of pathway involved in the uPA/uPAR signalling. Through its amino terminal fragment (ATF), uPA can bind to domain (D) 1 of its receptor uPAR. Via D2, D3, uPAR can interact vitronectin and members of the integrin family. Through its glycophosphatidyl inositol (GPI) anchor on D3, uPAR is associated to cell membrane. Collectively uPA/uPAR interaction can activate a number of key intracellular signalling pathways to 1) activate latent growth factors, 2) activate proteases, 3) promote tumor cell invasion, adhesion and migration, 4) facilitate matrix degradation and 5) promote angiogenesis.

6. Transcriptional regulation of uPA

Cancer development and progression to the metastatic stage involve the coordinated activation and deactivation of many specific genes. For a long time, cancer was regarded as primarily a genetic disease, with mutation in the DNA sequence being ascribed as the cause for the change in gene expression throughout cancer progression. However, it has now been established that epigenetic changes may also play a key role in the differential gene expression in

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cancer [94]. The epigenome is dynamic, with some parts of the epigenome being inherited or established during embryonic development, while other aspects are in a state of flux throughout life [95,96].

Epigenetic modifications can be made through various methods, including DNA methylation, nucleosome positioning, post-translational modification of histone tails, and non-coding RNA [97]. The protein machinery which is responsible for implementing these modifications consists of methyl-DNA binding proteins (MBDs), DNA methyltransferases (DNMTs), chromatin remodeling complexes, histone modifiers, and proteins which interact with histone modifications [95]. One of the most closely studied aspects of epigenetics is DNA methylation.

We were the first to identify the epigenetic regulation of uPA by examining the correlation between hormone (estrogen) sensitivity and expression of uPA in normal human mammary epithelial cells (HMEC), early stage hormone-responsive breast cancer cells lines (MCF-7 and T-47D), and late stage hormone-insensitive breast cancer cells (MDA-MB-231). uPA expression was only observed in the highly invasive MDA-MB-231 cells. Expression of various members of the PA system is shown in different human breast cancer cell lines in Table 1. Upon examination of the DNA methylation status of the uPA gene via Southern blot analysis using methylation sensitive enzymes, it was observed that CpG islands within the uPA gene are methylated in normal breast cells and early stage breast cancer cells. Conversely, the CpG islands of the uPA gene are hypomethylated in the highly invasive breast cancer cell line. Treatment of early stage MCF-7 cells with 5′ azacytidine (5-aza-C), a cytosine DNA methyltransferase inhibitor, caused demethylation of the uPA CpG islands and a dose-dependent expression of uPA mRNA [98]. Thus, this study was the first to demonstrate that expression of uPA in invasive vs. non-invasive breast cancers is regulated by DNA methylation of CpG islands within the gene and that this regulation is reversible. In another study conducted by us, methylation-sensitive PCR was used to quantify the methylation status of the CpG islands in the uPA promoter, comparing non-invasive hormone-sensitive MCF-7 cells to highly-invasive hormone-insensitive MDA-MB-231 cells. 90% of the CpG islands in the uPA promoter were found to be methylated in the MCF-7 cells, whereas the MDA-MB-231 cells had fully demethylated CpGs. Luciferase reporter assays demonstrated that the Ets-1 transcription factor binding, which regulates uPA promoter activity, was inhibited by methylation [99]. In order to determine the cause of the differences in the methylation status of the uPA promoter between MCF-7 and MDA-MB-231 cells, our group examined the levels of DNA methylation machinery. Both maintenance DNMT (DNMT1) and DNA demethylase (DMase) activities were shown to correlate with the methylation status of the uPA gene. Thus, MCF-7 cells show high DNMT1 activity and low DMase activity, resulting in a methylated uPA promoter, whereas MDA-MB-231 cells show increased DMase activity and reduced DNMT1 activity, resulting in a demethylated uPA promoter. DNA methylation was confirmed as the dominant mechanism in the silencing of the uPA gene, as histone deacetylase inhibitor Trichostatin A induced uPA expression in MDA-MB-231 cells but not in MCF-7 cell [99]. Thus, this study collectively demonstrated that DNA methylation is critical in the regulation of uPA expression in breast cancer cells.

Cell lines	Early Stage				Late Stage		
	MCF-7	BT-474	ZR 75-1	T-47-D	MDA-MB-231	BT-549	HS-578T
Invasion	-	-	-	-	+	+	+
ER	+	+	+	+	-	-	-
PR	+/-	+/-	+/-	+/-	-	-	-
HER2	-	+	+	-	-	-	-
uPA	N/D	N/D	N/D	N/D	High	High	High
uPAR	Low	Low	Low	Low	High	High	High
PAI-1	N/D	N/D	N/D	N/D	High	High	High
PAI-2	Low	Low	Low	Low	High	High	High

Expression of members of the plasminogen activator (PA) system, urokinase-type plasminogen activator (uPA), its receptor (uPAR), PA inhibitor 1 (PAI-1] and 2 (PAI-2] in human breast cancer cell lines (MCF-7, BT-474, ZR-75-1, T-47-D, MDA-MB-231, BT-549, HS-578T). uPA and PAI-1 are only detectable in highly invasive, estrogen (ER), progesterone (PR) receptor and Her-2 negative human breast (MDA-MB-231, BT-549, HS-578T) cancer cell lines.

N/D: None detected

Table 1. Expression of members of the plasminogen activator (PA) system in human breast cancer cells.

In a later study, our group set out to test the hypothesis that hypomethylation of the uPA promoter plays a causal role in breast cancer metastasis. In order to test this hypothesis, highly invasive MDA-MB-231 breast cancer cells were treated with different doses of the methyl donor S-adenosyl-methionine (SAM) for six days. SAM has been shown to inhibit hypomethylation, either through the inhibition of active demethylation or through the enhancement of DNMT activity [100]. Treatment with SAM resulted in a marked inhibition of uPA mRNA expression, accompanied by the expected decrease in uPA enzymatic activity [101]. Reduction in uPA production was accompanied by a significant decrease in tumor cell invasive capacity as determined by Matrigel invasion assay. The methylating capacity of SAM in breast cancer cells was confirmed, as the SAM-treated cells showed hypermethylation of the uPA promoter. Subsequent treatment of the SAM-treated cells with demethylating agent 5-aza-C caused a reversal of the observed uPA silencing, demonstrating that the effect of SAM on uPA expression is mediated through promoter hypermethylation. In *in vivo* studies carried out in immune deficient mice, animals were injected with MDA-MB-231 cells treated with vehicle or SAM via mammary fat pad. Experimental animals inoculated with MDA-MB-231 cells treated with SAM showed the development tumors which were significantly smaller in volume as compared to control animals. These anti-tumors effects of SAM were accompanied by a significant decrease in the development of tumor cells metastatic ability, resulting in significantly fewer

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metastatic foci in lungs, liver, kidney, spleen and kidneys as compared to animals inoculated with control cells (Figure 3). Analyses of tumoral RNA demonstrated that the tumors derived from SAM-treated breast cancer cells expressed no detectable levels of uPA, while uPA mRNA was highly expressed in tumors derived from control breast cancer cells. Thus, this was the first report to describe a potential epigenetic based strategy to block the expression of pro-metastatic genes like uPA which resulted in decreased tumor growth and metastasis [101].

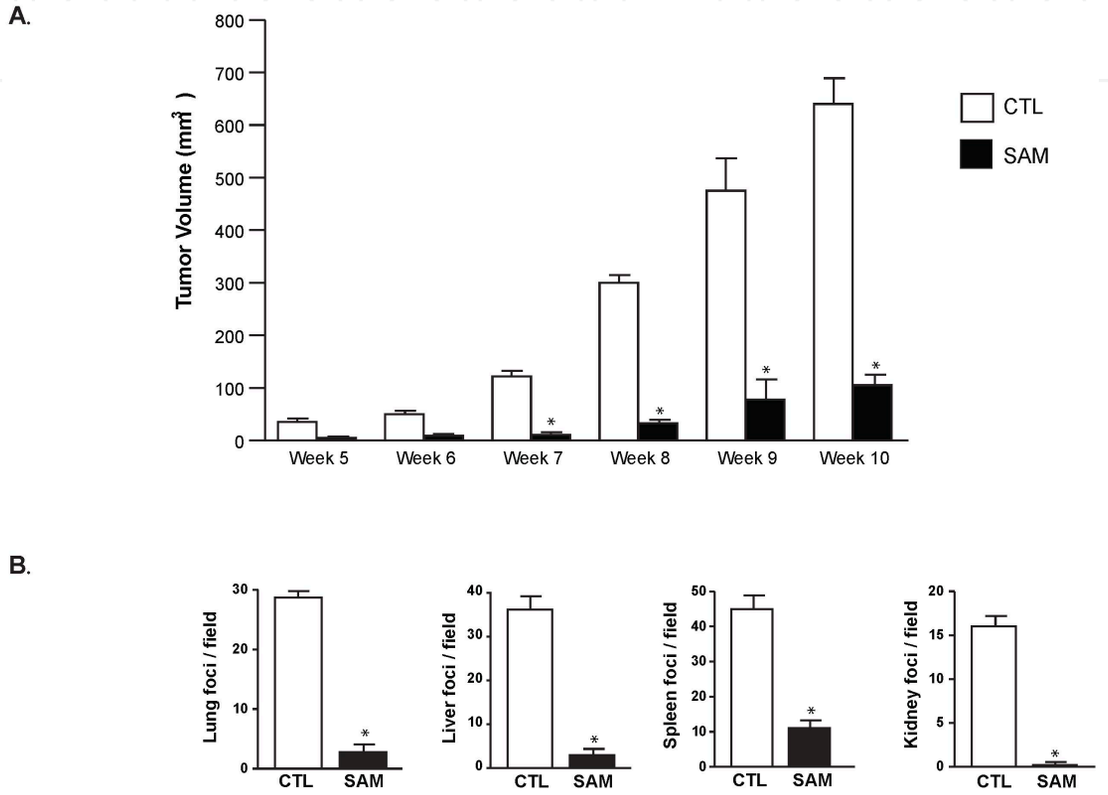

Figure 3. Effect of SAM on MDA-MB-231 tumor volume and metastasis. A: MDA-MB-231 cells treated with vehicle alone as control (CTL) or SAM were introduced into the mammary fat pad of female BALB/c nude mice. Tumor volumes were determined at weekly interval. **B**: At the end of these studies animals were sacrificed and fluorescent microscopic tumor foci in lungs, liver, spleen and kidneys were counted and compared with control group of animals. Significant difference from control is shown by an asterisk ($P < 0.05$). (Adapted from Pakneshan P et al; Ref. 101)

Demethylation results in the activation of tumor suppressor genes, which has led to development of demethylating agent 5-aza-C (Vidaza) for myelodysplastic syndromes, and which is now being tested for its beneficial effects in solid tumors [102,103]. The anti-tumor effects of SAM led us to investigate whether combining 5-aza-C and SAM can have additive or synergetic effects by activating tumor suppressor genes and suppressing pro-metastatic genes. Using several human breast cancer cell lines we have recently shown that SAM inhibits global and gene specific demethylation, prevents potential activation of pro-metastatic genes like uPA and MMPs, and potentiates the activation of tumor suppressor genes by 5-aza-C. These results have led us to propose epigenetic based demethylation (5-aza-C) and methylation (SAM) based therapies at different stage of tumor progression [104].

While a large number of these studies were carried out in breast cancer, DNA methylation has also been shown to regulate uPA and PAI-1 expression in prostate cancer, laryngeal squamous cell carcinoma, meningioma, and gastric cancer, where these genes are also identified as epigenetic based prognostic and therapeutic targets [105,106]. However, large scale clinical studies still remain to be carried out to demonstrate the impact of uPA-PAI-1 methylation in cancer. These epigenetic based therapies can also influence the effects of radiotherapy and chemotherapeutic agents to alter metastatic behaviour [107,108].

7. Diagnostic approaches

The field of cancer research has moved away from the development of broad drug classes which aim to target all cancers, and is instead moving towards personalized medicine. The current goal is to subdivide patients into groups based on molecular characteristics, which then allows therapy options to be assessed and administered based on the molecular characteristics within that particular group [109]. The proteins uPA and PAI-1 are now clinically used biomarkers which are unique among cancer biomarkers because of the lack of contradictory evidence which exists. This is especially surprising, given the variety of demographics which are covered by uPA/PAI-1 diagnostic studies [110]. Notably, uPA and PAI-1 have achieved the highest LOE-1 score attainable according the Tumor Marker Utility Grading System. uPA/PAI-1 are the only breast cancer biomarkers to reach LOE-1 [111].

In 1985, the first comprehensive report examining uPA expression in breast cancer was published. O'Grady *et al.* measured uPA proteolytic activity in both benign tumors and primary breast cancer tissue. Although no measurement was made of actual uPA antigen levels, the study demonstrated significantly elevated levels of uPA enzymatic activity in malignant tumors as compared with benign tumors [112]. In 1988, Duffy *et al.* added further to this area of research, showing that elevated levels of uPA proteolytic activity in primary cancer tissue was correlated with shorter disease-free intervals [113]. Later on, Jänicke *et al.* were first to examine actual proteins levels of uPA in breast cancer tissue, and in 1989 published a study which used the immunoenzymometric test ELISA, showing significant correlation between elevated expression of the uPA antigen in primary tumor tissues and poor prognosis of breast cancer patients [114]. Later on, the same group found a similar correlation existing for the uPA inhibitor PAI-1 [115]. In 2007, uPA and PAI-1 were added to the Breast Cancer Treatment Guidelines of the ASCO as novel cancer biomarkers. They are now used to help determine appropriate adjuvant systemic therapies in primary breast cancers [116].

Today, ELISA remains the gold-standard for assessment of uPA/PAI-1 correlation with breast cancer outcomes. It is the only system examining uPA/PAI-1 in which clinically relevant, validated data have been obtained. When conducting ELISA analysis, either detergent-released tumor-tissue fractions or tumor-tissue cytosolic fractions can be used [117]. Analysis can be conducted on core needle biopsies, primary tumor biopsies, and cryostat sections [118]. Therefore, a major advantage of the use of ELISA tests is the requirement for only very small tissue extract samples [119]. Currently, there is a commercially available ELISA-based assay

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called FEMTELLE® which is used to assess the probability of breast cancer reoccurrence in newly diagnosed women with node-negative breast cancer. FEMTELLE classifies women into categories of high or low risk of reoccurrence, based on the quantitatively-determined levels of uPA and PAI-1 found in tumor-tissue extracts [52].

A major disadvantage of FEMTELLE, and other ELISA-based assays, is the requirement of fresh or fresh-frozen tissue samples [109]. Thus, other methods of uPA/PAI-1 quantification are under investigation for validation. Immunohistochemistry allows the use of fixed, archived, paraffin-embedded tissue samples for analysis. A roadblock in the development of this assay is that uPA and PAI-1 are present in both tumor and stromal cells, as well as being released into the tissue. Thus, it is extremely difficult to develop a reliable scoring system for uPA/PAI-1 in immunohistochemical analysis. Nevertheless, in 1990 Jänicke *et al.* published a comparison of uPA levels obtained using immunohistochemical scoring and ELISA. The study showed a statistically significant increase in staining intensity for uPA in immunohistochemistry which correlated with an increase in ELISA uPA values [120]. Reilly *et al.* later published the same correlation for PAI-1 [121]. Thus, much work is being done to develop immunohistochemistry as a validated, clinically relevant method of quantifying uPA/PAI-1 expression in breast cancer samples. It is important to note that significant correlation is yet to be established between plasma levels of uPA/PAI-1 with tissue expression of these proteins. Thus, expression of uPA/PAI-1 must be measured directly in the breast cancer tissue sample, and cannot be extrapolated from any plasma measurements [122].

Rather than measuring protein expression levels of uPA and PAI-1 in breast cancer tissue, much research is also invested in the assessment of uPA and PAI-1 biomarker expression at the transcriptional level. The highly sensitive quantitative reverse transcription-polymerase chain reaction (RT-PCR) does not require fresh or fresh-frozen tissue samples, as it can use formalin-fixed tissue specimens and only requires minute amount of mRNA for assessment [123]. Significant correlation between transcript and protein levels for uPA and PAI-1 have been found in breast cancer cell lines [123]. Unfortunately, no correlation was found when examining breast cancer tissue specimens. Spyratos *et al.* found no significant correlation when examining uPA expression, and found only a weak correlation in the case of PAI-1 [124]. Conversely, Lamy *et al.* was able to show high concordance between uPA/PAI-1 antigen expression, as assessed by ELISA, and mRNA expression as assessed by the novel technique nuclei acid sequence-based amplification (NASBA) [125]. However, the results of this study require future validation.

The final area of study which examines the correlation between uPA/PAI-1 expression and breast cancer prognosis is the examination of DNA methylation. As this is a DNA-based assessment, this form of analysis can be easily carried out in formalin-fixed, paraffin-embedded samples, using PCR-based or DNA array technology [109]. As described above, our lab demonstrated the correlation between uPA promoter methylation status in breast cancer [98]. In this study uPA promoter was methylated in normal mammary epithelial cells and in low invasive breast cancer cell lines. In contrast the uPA promoter was demethylated resulting in high levels of uPA expression. Using surgical biopsy specimens, uPA promoter demethylation was associated with advanced disease stage (Figure 4). This effect was independent of the

hormone receptor status and results from this study demonstrated the determination of uPA promoter methylation can be developed as a reliable and early marker for uPA expression in breast cancer patents [126]. A similar correlation has also been demonstrated for the PAI-1 promoter [105]. Using surgical biopsy specimens from breast cancer patients, we demonstrated a correlation between uPA promoter methylation status and disease stage correlating with uPA mRNA expression which can serve as an early and reliable diagnostic and prognostic marker for breast cancer [126].

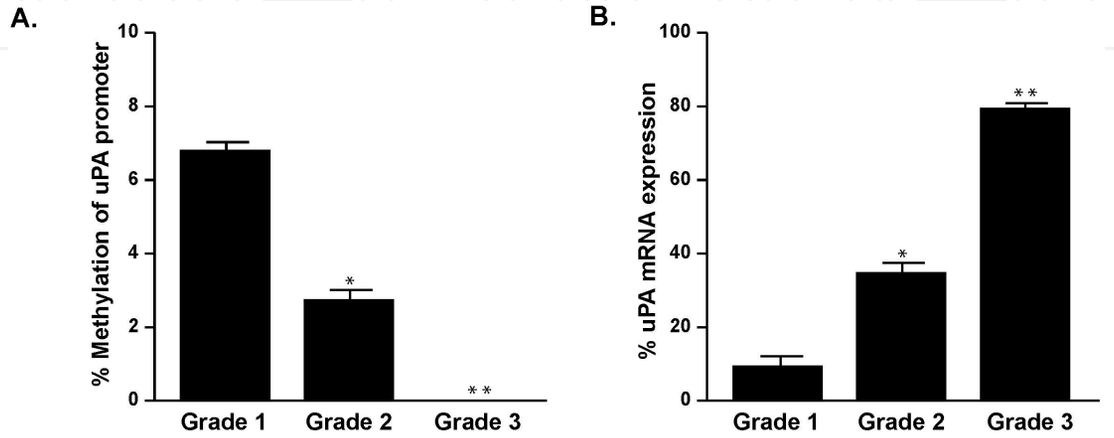

Figure 4. Reverse correlation between percentage of methylation of the urokinase promoter (uPA) and uPA mRNA expression in breast cancer. Percentage of methylation of the uPA promoter (A) and the uPA mRNA expression (B) in the biopsy samples of breast cancer patients were analyzed and graphed. Results are the mean ± SE of at least three independent analyses. Significant difference from grade 1 is shown by an asterisk, and significant difference from both grade 1 and 2 is shown by two asterisks ($P < 0.05$). (Adapted from Pakneshan P et al; Ref. 126)

8. uPAR as an imaging target in breast cancer

Continued development of novel targeted therapies and the effective use of current approaches for breast cancer are still not yielding optimum benefit due to poor strategies to monitor therapeutic efficacy. While diagnostic imaging is extensively used to stage cancers and assess therapy effectiveness, development of highly sensitive non-invasive imaging agents which can identify aggressive lesions while also identifying residual disease will prove to be highly beneficial. High levels of uPAR in cancer lesions as compared to adjacent tissue and normal hemostatic tissues provide a unique opportunity to target uPAR as an imaging target in several common malignancies [127-129]. These unique characteristics allowed the development of non-invasive approaches to detect invasive cancers and detect the presence of occult tumor metastases [130-132]. Our group was first to identify uPAR as an imaging target in cancer and towards these goals we used our well established syngeneic model of breast cancer, which led to the validation of uPAR as a viable target to detect the presence and progression of cancer [133]. In a series of studies, a species specific (rat) antibody directed against the rat (r)-uPAR was developed and characterized by immunofluorescence and Western blot analysis. Following ^{125}I-labelling of the antibody, the binding of r-uPAR-IgG was confirmed in rat

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prostate cancer cells (Dunning R3227 Mat Ly Lu) and breast cancer cells (Mat B-III) overexpressing (r)-uPAR (Mat B-III-uPAR). In *in vivo* studies, ^{125}I-rat (r)-PAR-IgG was injected on to rat breast and prostate cancer tumor-bearing animals. Uptake of this radiolabel was seen in primary tumors and in liver, spleen, lungs, and lymph nodes, which are common sites of tumor metastasis in these models. Minimal levels of radioactivity were seen in these organs in normal animals and tumor-bearing animals injected with ^{125}I-labeled pre-immune IgG. This study not only further confirmed uPAR as a therapeutic target but also validated it as an imaging target to monitor tumor progression and metastasis.

Following our report, a number of groups have actively pursued these goals; uPAR is now established as an excellent imaging target in cancer. Studies in this regard include the use of dual labelled nanoparticles conjugated to the ATF of uPA, which allowed the accumulation of dye in a xenograft model of pancreatic cancer [134]. Following its internalization, the use of nanoparticles was shown to increase dye retention in the primary tumor and metastatic sites.

The organic compound, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (also known as DOTA) has medical application including its use as an imaging agent. DOTA was conjugated to the lead uPAR-targeted peptide (AE-105) and labelled with ^{64}Cu [135-137]. It was successfully shown to monitor the levels of uPAR-expressing tumor cells using positron emission tomography (PET) in a human glioma xenograft model. In this study, solid-based synthesis was carried out via Fmoc approach, followed by the elution and concentration of chelator used for labelling. The labelled reagent was characterized in a series of *in vitro* studies to determine its uptake followed by dynamic ET imaging in tumor-bearing mice. Use of gallium (Ga) based tracers and PET imaging with targeting peptide was shown to be highly effective due to its high radiochemical yield, purity, stability, cellular uptake and good tumor to background ratio using non-invasive PET-based imaging which will be highly useful in a clinical setting [135]. These investigators followed up by combining their findings with a therapeutic approach as well where AE105 was first labelled with ^{64}Cu and ^{177}Lu for its uses in PET-based imaging as well as radionuclide therapy in a xenograft model of colorectal cancer [136].

Various imaging modalities like plane film X-ray, bone scan, ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and PET are used alone or in combination. PET is a non-invasive imaging technique that offers substantial advantages over anatomic imaging modalities in oncology. Additionally, PET can often distinguish between benign and malignant lesions. Given that highly expressed receptors like uPAR or enzymes can be linked to prognosis in many cases, targeted imaging with highly specific probes may provide prognostic information concerning the level of differentiation of breast cancer, both at primary and metastatic sites.

Over the past few years, there has been a significant growth in the development of radiolabeled monoclonal antibodies (mAbs), which bind with high affinity to receptors frequently highly overexpressed on diverse human cancer cells, for diagnostic and therapeutic applications [138,139]. Characterization of the structure of ATN-658 and its demonstrated efficacy in several xenograft models has led to the initiation of clinical trials using ATN-658 as a therapeutic agent [140]. Availability of this selective anti-uPAR antibody provides us with the opportunity to

evaluate it as an imaging agent using multiple radiolabels which can be effectively used to develop PET tracers.

Collectively, evidence continues to accumulate in multiple models, validating uPAR as a viable imaging target for future translational studies for the use of uPAR imaging agents in patients with various malignancies, where overexpression of uPA-uPAR system plays a major role in tumor progression.

9. Targeting the PA system in breast cancer

Since first identifying the PA system as an important player in breast cancer progression and metastasis, there have been many attempts made to target this system specifically. Early development focused on the inhibition of plasminogen activation, looking to inhibit uPA enzymatic activity. This could be accomplished either through the use of small molecules to block the active site of uPA, or by attempting to block the binding of uPA or scuPA to uPAR. Blocking the binding of uPA to uPAR proved to be a more challenging method, as uPAR's binding pocket is much larger than the enzymatic active site of uPA [56]. Many studies have been published which show early attempts at blocking proteolytic activation of plasminogen by uPA.

A common approach was to use small-molecule inhibitors of uPA to block its enzymatic activity, thereby reducing proliferation, invasion, and metastasis. Using this strategy we showed the use of one such uPA inhibitor (B-428) for its ability to block tumor metastases in a xenograft model of prostate cancer. Infusion of B-428 into syngeneic rats inoculated with rat (r) prostate cancer cells Mat LyLu which overexpressed r-uPA resulted in a significantly decreased tumor volume and smaller metastatic foci, as compared with control tumor bearing animals receiving vehicle alone [141]. Other serine protease inhibitors have also been used, and have even been advanced into clinical trials. Promising results have recently been reported with regard to a Phase Ib clinical trial using serine protease inhibitor WX-UK1 for treatment of breast cancer, as well as other solid tumors [142]. A similar agent, known as WX-671 (MESUPRON®), which is a pro-drug of WX-UK1 has also completed a Phase Ib trial for treatment of patients with head and neck cancer [143]. MESUPRON has now moved on to two Phase II clinical trials, currently underway, in which it is being given patients with advanced breast or pancreatic cancer. In both trials, patients are receiving MESUPRON alongside a traditional chemotherapy drug, Capecitabine and Gemcitabine for breast and pancreatic cancer, respectively [5].

Other methods which have been used to successfully block plasminogen activation through inhibition of the uPA system include peptide inhibitors of the uPA-uPAR interaction and anti uPA-uPAR antibodies [144,145]. A non-competitive antagonist of the uPA-uPAR interaction corresponding to the amino acid 136-143 was identified and this peptide (A6) was shown to inhibit endothelial cell migration and breast cancels invasion *in vitro* [146]. Treatment of breast cancer cells MDA-MB-231 tumor-bearing mice resulted in significant inhibition of tumor volume and metastasis (Figure 5). These experimental tumors also showed decreased factor

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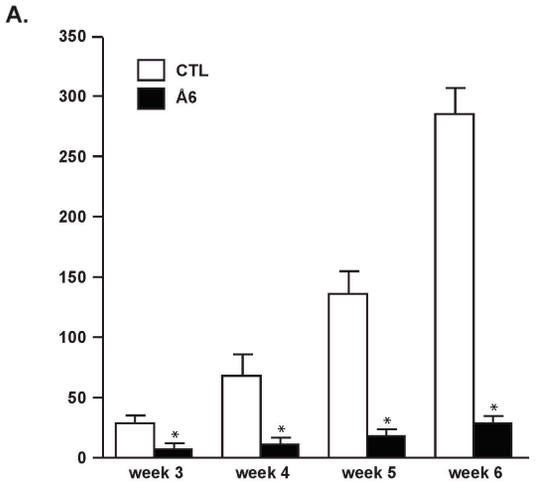
VIII-positive tumor micro vessel hot-spots, establishing the anti-angiogenic effects of A6. In studies carried out by Mishima *et al.* the antitumor and anti angiogenic effects of A6 were shown alone and in combination with chemotherapeutic agent Cisplatin in a glioblastoma model which led to the clinical evaluation of A6 [147,148]. Use of antibody based therapies has been established during the last decade, resulting in highly beneficial therapeutic approaches for various cancers [149]. The use of antibodies to block uPA-induced metastasis has met with some success, as described below. Using a polyclonal anti-rat uPAR antibody we targeted the ligand binding NH_2-terminal domain of rat uPAR we showed its ability to block breast cancer growth and metastasis *in vivo* [133]. More recently, we evaluated the potential of a highly selective monoclonal antibody against human uPAR (ATN-658). First we examined the efficacy of ATN-658 in blocking prostate cancer growth, invasion, migration, and skeletal metastasis. Examination of the effects of ATN-658 administration *in vitro* using human prostate cancer PC-3 cells showed its ability to cause a decrease in tumor cell invasion and migration by interference with downstream signaling molecules involved in mediating the effects of uPAR (Figure 6). In *in vivo* studies ATN-658 administration caused a significant decrease in tumor volume and number of skeletal metastatic foci [150]. Using ATN-658, Larengyl *et al.* showed its ability to block ovarian cancer metastasis by inducing apoptosis and u-PAR-α_5-integrin interaction [151]. Recently, we have examined the effect of ATN-658 alone and in combination with the bisphosphonate Zometa on skeletal metastasis associated with breast cancer. ATN-658 had a significant effect on reducing the number and area of skeletal lesions as determined by X-ray, however these effects were more pronounced when ATN-658 and Zometa were administered in combination (Rabbani *et al*., unpublished observations).



B.

	TISSUE	CONTROL	Å6
Macroscopically (number of tumor foci)	LUNG	6 ± 2	3 ± 2
	AX. LYMPH NODES	5 ± 2	1 ± 1
Microscopically (tumor cells per field)	LUNG	4.23 ± 0.18	2.09 ± 0.30
	LIVER	18.83 ± 0.36	3.11 ± 0.14
	SPLEEN	35.67 ± 0.82	28.30 ± 2.80

Figure 5. Effect of Å6 on tumor growth and metastases. A: MDA-MB-231-GFP tumor-bearing BALB/c (nu/nu) mice were injected i.p. with Å6 or vehicle alone (CTL) and tumor volume was determined at weekly intervals. **B**: At the end of this study, control and experimental mice were sacrificed to count the number of macroscopic and microscopic fluorescent tumor foci in different organs. Significant difference from control tumor-bearing animals after treatment with Å6 is denoted by asterisks ($P<0.05$). (Adapted from Guo Y et al; Ref. 146)

Additional efforts towards therapeutic targeting of the PA system in breast cancer have focused on either decreasing uPA/uPAR/PAI-1 expression, or have focused on using uPA/

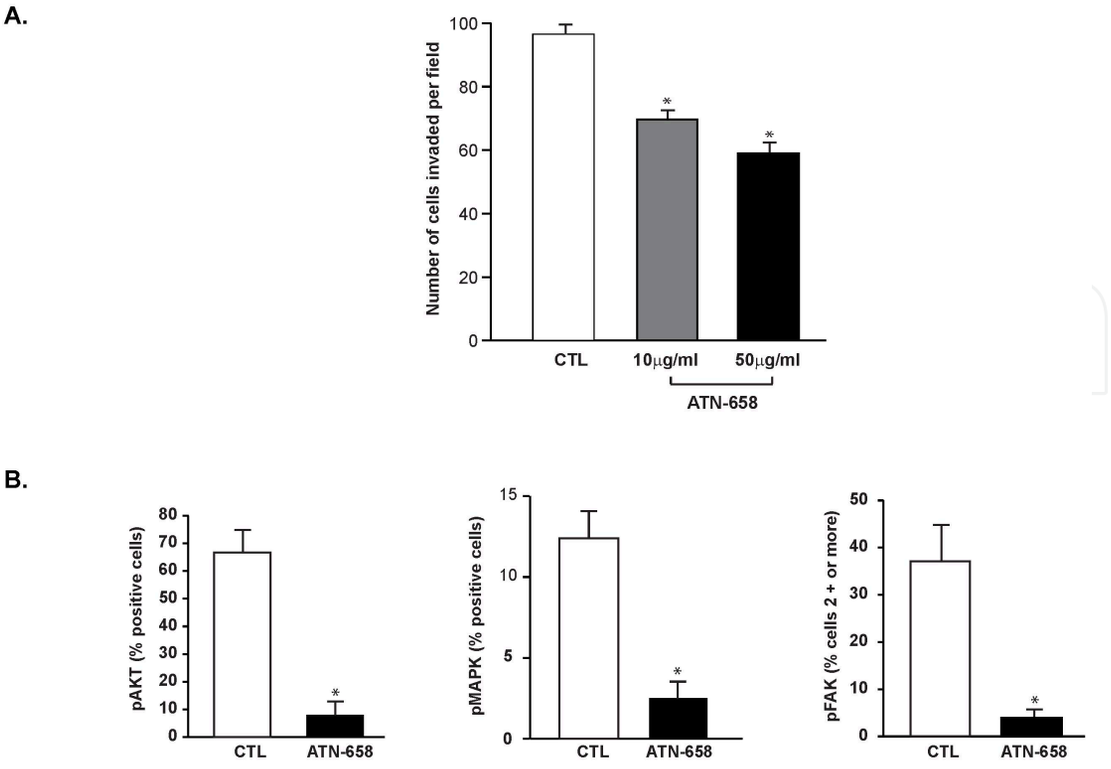


Figure 6. Effect of ATN-658 on tumor cells invasion in vitro and intracellular signaling pathways *in vivo*. A: Human prostate cancer cells PC-3 cell invasive capacity was evaluated after treating with control IgG or ATN-658 using a Boyden chamber Matrigel invasion assay. Number of cells invading is shown as bar diagram ± SEM. B: Male Fox chase SCID mice were inoculated with PC-3 cells through the intra tibial route of injection. Animals were treated with 10.0 mg/kg of control IgG (CTL) or ATN-658. At the end of these studies, animals were sacrificed, and tibias were removed, formalin-fixed, and subjected to immunohistochemical analysis to determine the effect on various intracellular signaling pathways. (Adapted from Rabbani SA et al; Ref. 150)

uPAR as homing mechanisms for cytotoxic drugs. Techniques which aim to reduce or block the expression of uPA/uPAR/PAI-1 include the use of antisense oligonucleotides, interference (RNAi), ribozymes, or DNAzymes [55,152-155]. Experiments using these techniques have shown significant effects on uPAR signaling and tumor behaviour. Anti-uPAR antisense oligonucleotides have been used to inhibit cancer cell proliferation and invasion *in vitro* using melanoma cells, while *in vivo* experiments also showed inhibition of tumor growth and metastasis [156]. Down regulation of uPA and uPAR expression using RNAi has also shown promise, and *in vitro* experiments using human glioma cells showed inhibition of pro-cancer signaling molecules, such as RAS-and MEK-mediated signaling, and resulted in activation of apoptosis [157]. As mentioned above, Pakneshan *et al.* have shown that treatment of highly invasive breast cancer MDA-MB-231 cells with SAM results in decreased expression of uPA, as well as decreased tumor proliferation, invasion, and metastasis [101]. While the exact mechanism through which SAM exerts its methylating actions is still being debated, SAM is a methyl donor and thus may increase the number of methyl groups available for the methyltransferase reaction [94]. SAM has also been shown to inhibit DNA demethylase activity, including MBD2 [100]. Thus, uPA/uPAR expression can be targeted at the transcriptional or at the translational level as well.

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Not only is the PA system an excellent therapeutic target because of its pro-metastatic effects, but it is also an exciting group of proteins because of the specificity through which it is highly expressed in tumor cells and the surrounding stroma. This allows for therapeutic targeting of cytotoxic drugs to the tumor compartment through the use of uPA-derived or other uPAR-binding peptides. One example is the conjugation of the growth factor domain (GFD) of uPA to the chelator DOTA and 213-Bi, an α-emitter. With the GFD portion binding to uPAR, this combination has been shown to be cytotoxic to uPAR-expressing ovarian cancer cells *in vitro* [158]. It is also possible to use the amino terminal fragment (ATF) of uPA to deliver drugs to the tumor compartment. ATF binds uPAR with an affinity similar to that of the full sized uPA peptide, resulting in extremely effective delivery of the ATF-conjugated therapeutic payload. Many ATF-toxin fusions have been made, including a ATF-pseudomonas exotoxin (PE), which has been shown to be effective against a number of cancerous cell lines, and ATF-diphtheria toxin (DTAT), which has shown efficacy both *in vitro* and *in vivo* [159-161].

Another recent area of exploration is the use of nanobins, a novel liposomal nanoparticle drug encapsulation and formulation system. Nanobins take advantage of the 'enhanced permeability and retention effect' (EPR effect), in which molecules of certain sizes tend to accumulate in tumor tissue more so than in normal tissue [162]. Although nanobins were already designed to target the tumor environment, relying either entirely on the EPR effect or in conjunction with the use of a pH-responsive cross-linked polymer shell, it is also possible to conjugate nanobin technology with uPA/uPAR-targeting techniques. O'Halloran *et al.* describe their current efforts to combine the monoclonal anti-uPA antibody ATN-291 with nanobins, creating a product which can be internalized into tumor and tumor-associated cells for greater therapeutic strength. ATN-291 binds to the kringle domain of uPA and is able to bind uPA which is already bound to uPAR. Interestingly, the internalization of the ATN-291-uPA-uPAR complex is not dependent on the presence of PAI-1. The efficacy of this system is currently being evaluated in several xenograft models, with hopes of advancing this technology into clinical development sometime in the near future [6].

One caveat when studying any uPA/uPAR-targeted therapy is the high degree of species specificity of uPA and uPAR, such that human uPA has an extremely low binding capacity towards murine uPAR, and vice versa. This is especially relevant to the use of xenograft models, in which therapies which target human uPA/uPAR will only have an effect on tumor cells, and not on the surrounding stromal cells [56]. One result of this issue is that the efficacy of potential uPA/uPAR-targeted therapies may be underestimated in xenograft models. The second implication is that the toxicity profiles of these drugs may also be underestimated in xenograft models. However, toxicity concerns can be somewhat put to rest, as analysis of cadaveric human tissue has demonstrated very little tissue expression of uPAR [7].

Like uPA, several studies have been carried out targeting the PAI-1 as an anti-cancer therapy. Elevated levels of PAI-1 are a predictor of aggressive cancers, although that fact seems contradictory, given that PAI-1 is an inhibitor of uPA activity. However, it is now believed that PAI-1 may possess functions independent of uPA inhibition [163]. For example, expression of PAI-1 is necessary for cancer-induced angiogenesis in preclinical models [164]. In addition, PAI-1 is associated with insensitivity to chemotherapy treatment, while PAI-1

deficiency causes increased chemotherapy sensitivity [165]. A way of targeting these actions is to inactivate PAI-1, forcing the conversion of PAI-1 into its latent form. This can be done using the small peptide paionin-4-D1D2 or small-molecule inhibitor PAI-039 [166,167]. Another method under examination is the interference of the interaction between PAI-1 and vitronectin, an interaction which has been shown to cause detachment of tumor cells from the ECM, promoting the metastatic process [168]. RNA-aptamers SM-20 and WT-15 are effective in inhibiting this interaction without affecting the uPA-inhibiting activity of PAI-1 [155, 169].

Thus, the PA system represents a promising area of research for the development of targeted anti-cancer therapies. There are a wide variety of methods being examined, targeting any of the three key players within the PA system, and using several molecular, chemical, and immunological approaches which have already shown highly promising results, paving the way for their clinical evaluation.

10. Summary and future goals

Within the last 20 years, the PA system has been established as an important regulator of breast cancer progress, being directly involved in proliferation, invasion, and migration of tumor cells. As such, it has become a key target for clinical use in diagnostics, imaging, and therapeutics. Over the next few years, there will likely be many more important developments in this field of study. The exact nature of the signalosome relationship is still being elucidated, and several studies are underway to identify which proteins are directly bound to uPAR and are involved in its intracellular signaling. Although ELISA is currently being used as the gold-standard in measuring uPA/uPAR for diagnostic purposes, much work is being done to establish immunohistochemical protocols, so that fresh or fresh-frozen tissue samples are no longer required. Much research is being conducted to evaluate the potential regulation of uPA/uPAR/PAI-1 expression via epigenetics as well as antisense oligonucleotides and RNAi. In addition, technologies which use uPA and uPAR to target cytotoxic drug to the tumor compartment are only now in their earliest stages of development, thus, there are many avenues to explore in that area of research. Collectively, results from these studies will drive the clinical development of several PA targeted diagnostic and therapeutic agents which are either already in clinical trials are expected to enter in the near future. There is great optimism in these studies using targeted approaches which will lead to reduced morbidity and mortality in several common malignancies, including breast cancer.

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