Serine Proteases and Serine Protease Inhibitors in Breast Cancer Cell Pathophysiology

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Abstract

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(Under the direction of Frank C. Church, Ph.D.)

Hemostasis and fibrinolysis are mediated by serine proteases and regulated by serine protease inhibitors, or serpins. There is a growing body of evidence that a few of these serine proteases and serpins have direct effects on cancer pathophysiology. Activated protein C (APC) is a serine protease that limits excessive clot formation by catalyzing inactivation of other coagulation factors. APC has also been shown to have non-hemostatic functions related to inflammation, angiogenesis, and cell migration. Therefore, we investigated the mechanisms by which APC could promote angiogenesis and breast cancer invasion. We confirmed that proteolytically active APC promotes angiogenesis by a mechanism dependent on matrix metalloprotease (MMP) activity and EGFR transactivation. Furthermore, we show that APC promotes breast cancer cell invasion by a similar mechanism and also show that APC induced cancer cell invasion is dependent upon activation of intracellular signaling pathways, specifically MAPK and PI3K signaling. Another serine protease, urokinase plasminogen activator (uPA), initiates fibrinolysis by activating plasmin – the protease which catalyzes degradation of fibrin clots. uPA activity is inhibited by the serpin plasminogen activator inhibitor 1 (PAI-1). Outside of fibrinolysis, uPA and PAI-1 are regulators of tumor cell motility through the focusing of their activities at the cancer cell surface. In breast

cancer, clinical studies have demonstrated that elevation of both uPA and PAI-1 in tumor biopsies is predictive of poor prognosis. Therefore, we utilized breast cancer cell lines to investigate a possible direct mechanism whereby elevation of both uPA and PAI-1 could provide a survival advantage to breast cancer cells. Utilizing inhibitors of uPA and PAI-1 activity, as well as stable knockdown of each factor individually, we demonstrate that uPA promotes cell growth via activation of MAPK signaling. We also demonstrate that the catalytic function of both uPA and PAI-1 promote cell growth. We found PAI-1 is a key regulator of both MAPK signaling and anti-apoptotic PI3K signaling. Finally, we found that PAI-1 protects breast cancer cells from apoptosis inducing chemotherapeutic drugs, suggesting a direct mechanism whereby PAI-1 elevation is associated with poor prognosis in breast cancer.

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List of Abbreviations

APC	Activated protein C
ATF	Amino-terminal fragment
BBE	Bovine brain extract
BME	Basement membrane extract
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
EPCR	Endothelial protein C receptor
ER	Estrogen receptor
ERK	Extracellular regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FN	fibronectin
GPCR	G-protein coupled receptor
GPI	glycophosphotidyl-inositol
HB-EGF	Heparin-bound epidermal growth factor
HER2	Human epidermal growth factor receptor 2
HUVEC	Human umbilical vein endothelial cells

IL	Interleukin
JNK	Jun N-terminal kinase
LOE	Level of evidence
LRP	Low density lipoprotein like receptor
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein-1
M-MLV	Mouse mammary leukemia virus
MMP	Matrix metalloprotease
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAI-1	Plasminogen activator inhibitor 1
PAGE	Pulsed applied gel electrophoresis
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PC	Protein C
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3 kinase
PKA	Protein kinase A
PKC	Protein kinase C
PR	Progesterone recptor
PS	Protein S
RNA	Ribonucleic acid
RNAi	Ribnonucleic acid interference

S1P	Sphingosine-1 phosphate
SDS	Sodium dodecyl sulfate
serpin	Serine protease inhibitor
shRNA	short hairpin ribonucleic acid
TACE	tumor necrosis factor alpha converting enzyme
TF	Tissue Factor
TGF	Transforming growth factor
TM	Thrombomodulin
TRAIL	TNF-related apoptosis-inducing ligand
tPA	tissue-type plasminogen activator
TNF	Tumor necrosis factor
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VN	Vitronectin
vWF	Von Willebrand factor

Chapter I

Introduction

Breast cancer is the second leading cause of cancer related death for women in the U.S., second only to lung cancer. A diagnosis of 207,090 new cases of breast cancer is predicted for 2010. Based on data gathered from 1996 to 2003, the five year survival rate for breast cancer that is localized at the time of diagnosis is approximately 98%. However, if at time of diagnosis breast cancer has spread regionally, the 5 year survival rate falls to 84%. In cases where distant metastases are present at time of diagnosis, the 5 year survival rate is very low – approximately 23%. Among women, breast cancer accounts for 15% of cancer related deaths, predicted to account for 40,730 breast cancer related deaths for women in 2010 [1].

Death cancer rates have declined steadily in the past 20 years in part through increased use of mammography for early detection. In addition, the development of targeted therapies in breast cancer to complement traditional chemotherapeutic regimens has improved treatment. The development of a monoclonal antibody targeting human epidermal growth factor receptor 2 (HER2) has proven effective in treatment of women with surgically removed and metastatic HER2 positive breast cancer [2,3]. The development of hormonal therapy for women with estrogen receptor (ER) and/or progesterone receptor (ER) breast cancer has also improved survival and recurrence rates [4,5]. The success of these targeted therapies highlights the need for continued research into novel factors that drive breast cancer pathophysiology. This is important for both identifying new prognostic indicators as well as potential targets for therapeutic intervention.

There is an emerging body of evidence that serine proteases and serine protease inhibitors, which regulate hemostasis (clot formation) and fibrinolysis (clot dissolution), also have functions in cancer physiology. More specifically, the hemostatic serine proteases

thrombin and activated protein C (APC) have been shown to regulate processes that can contribute to cancer growth and metastasis, including angiogenesis, cancer cell motility, and tumor growth and resistance to apoptosis. The serine protease urokinase-type plasminogen activator (uPA), which initiates fibrinolysis, also has been implicated in promoting cancer cell growth and metastasis. Intriguingly, there is some evidence which suggests the serine protease inhibitor plasminogen activator inhibitor 1 (PAI-1), which inhibits uPA, also contributes to tumor growth and metastasis. Based on these observations, in this study we closely examined the roles of PAI-1, APC, and uPA in breast cancer cell pathophysiology.

In Chapter II, I review the literature on the role of the serine proteases thrombin, APC, and uPA in cancer pathology. Thrombin, which in the vasculature initiates clot formation, has demonstrated pro-angiogenic functions and can also stimulate tumor cell motility – capacities that contribute to metastasis. APC, which inhibits clot formation, has activities related to cancer pathology that are either parallel or opposite to thrombin, depending on experimental context. This is intriguing given that thrombin and APC target the same cell receptor on endothelial cells and cancer cells. Multiple studies have shown that uPA coordinates a wide array of activities that contribute to tumor growth and metastasis.

Chapter III is an examination of the mechanisms by with the serine protease APC could contribute to breast cancer biology [6]. We demonstrate that APC promotes angiogenesis using *in vitro* and *ex vivo* models. Our findings also show that the APC promotes cancer cell invasion by a mechanism far more complex than suggested in previous reports. We show that APC stimulates breast cancer cell motility and invasion via a mechanism that is dependent upon both extracellular protease activity and the activation of intracellular signaling pathways.

Chapter IV is a review of the pleotrophic activities of the serine protease inhibitor PAI-1 in the pathophysiology of various diseases [7]. PAI-1 has been implicated in playing a role in diseases such as atherosclerosis, restenosis, and cancer. For some disease states, the capacity of PAI-1 to inhibit the serine protease uPA seems to fully explain its role. However, in other cases, the protease inhibitor capacity of PAI-1 does not seem sufficient to explain its role. Therefore, we review recent evidence suggesting PAI-1 can also participate in processes related to motility, proliferation, and sensitivity to apoptosis.

In Chapter V, I examine the contributions of both PAI-1 and uPA to the viability of breast cancer cells. Basically, this study is a focused attempt to address a long standing paradox in the field, known as the "PAI-1 Paradox." Multiple studies have indicated that elevation of both uPA and PAI-1 are predictors of lower disease survival, increased recurrence, and increased metastasis. The paradox arises because it is unclear why elevated PAI-1 levels would be associated with metastasis if the primary role of PAI-1 is only to inhibit the proteolytic events catalyzed by uPA. We confirmed uPA promotes cell growth and proliferative signaling, as shown by others in other forms of cancer. Uniquely, we also show that PAI-1 promotes proliferative and anti-apoptotic signaling as well as protects breast cancer cells from apoptosis inducing chemotherapeutic agents.

Finally, in Chapter VI, I address the significance of our findings to breast cancer pathophysiology and discuss future directions to expand upon these findings.

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Chapter II

Thrombin, Activated Protein C, and Urokinase Plasminogen Activator:

The role of coagulation serine proteases in cancer pathophysiology

Manuscript submitted for publication

Abstract

The serine proteases thrombin, activated protein C (APC), and urokinase-type plasminogen activator (uPA) are well documented regulators of hemostasis and fibrinolysis via their canonical targets in the coagulation and fibrinolytic pathways. Research into activities of these proteases outside of hemostasis-fibrinolysis has revealed their capacity to participate in key processes in cancer pathophysiology. For thrombin and APC, cleavage of transmembrane protease-activated receptors (PARs) has been demonstrated to activate intracellular signaling pathways that up-regulate various growth factors and cytokines to promote angiogenesis. Thrombin and APC have also been shown to directly activate signaling pathways that promote growth and motility of both endothelial and cancer cells. uPA, in conjunction with its cognate cell surface receptor uPAR, has a broad capacity to regulate matrix remodeling, which has implications for both angiogenesis and cancer cell motility. Like thrombin and APC, uPA can also directly activate intracellular signaling pathways in cancer cells that promote growth and motility, albeit by different mechanisms. In the tumor microenvironment, the interplay of these three proteases has the potential to greatly impact cancer cell growth and metastasis.

Introduction

The serine proteases thrombin, activated protein C (APC), and urokinase-type plasminogen activator (uPA) are known for their role in regulating hemostatic and fibrinolytic pathways. Thrombin catalyzes the culminating event in clot formation via cleavage of fibrinogen to form fibrin, which polymerizes to form a hemostatic plug (clot). APC, which is activated from its zymogen form protein C (PC) by thrombin bound to thrombomodulin (TM), provides a negative feedback signal to prevent excessive clot formation by its proteolytic inactivation of factors Va and VIIIa. uPA, similar to its counterpart tissue plasminogen activator (tPA), initiates fibrinolysis by cleaving plasminogen into plasmin, which catalyzes the degradation of fibrin to fibrin degradation products.

More recently, characterization of molecular targets of these proteases outside of hemostasis and fibrinolysis has revealed their broad capacity for participating in various physiological and pathophysiological processes. Discovery of the GPCR protease activated-receptor-1 (PAR-1) has revealed direct cell signaling activated by thrombin and APC through cleavage of PAR-1 in multiple cell types. Outside of its capacity to activate fibrinolysis, uPA is capable of initiating proteolytic cascades that drive extracellular matrix remodeling. All three of these serine proteases have been shown to activate or induce growth factors to promote angiogenesis, directly promote cell motility, and in some cases act as mitogens. The diverse, and at times convergent, pathways regulated by these coagulation proteases have profound implications in cancer. This review aims to briefly address the potential mechanisms whereby these proteases can participate in cancer pathophysiology – notably with regard to cancer cell proliferation and motility, angiogenesis, and metastasis.

Thrombin

When a vessel is injured, the extrinsic pathway of blood coagulation is initiated by the exposure of tissue factor (TF) to blood. TF is a transmembrane receptor that is expressed on epithelial cells, smooth muscle, and other cell types and is not typically exposed to blood [1,2]. The TF-factor VIIa complex activates factor X to Xa and factor IX to IXa. Factor Xa activates prothrombin to thrombin [Reviewed in 3, 4]. Thrombin subsequequently catalyzes the cleavage of fibrinogen to fibrin and is also capable of activating platelets via cleavage of PAR-1 and PAR-4 [5]. Many have shown that TF is expressed on tumor-associated endothelial cells [6], stromal cells surrounding the tumor [7], and the tumor cells themselves [8]. In this way cancer can activate thrombin, which has downstream effects on tumor growth and metastasis mainly through the effects of thrombin on its primary target PAR-1. PAR-1 is capable of activating multilple signaling pathways via its documented coupling to multiple G protein families [Reviewed in 9].

Correlations between thrombin activity and cancer development/metastasis.

Multiple studies have demonstrated direct linkages between thrombin activity and cancer as well as linkages between thrombin activity and the proclivity of cancer to metastasize. Thrombin activity is elevated in peripheral blood in patients with glioma [10] as well as multiple blood borne myeloproliferative neoplasms [11]. For lung cancer, elevated thrombin activity in blood is associated with greater extent of disease in small cell and non-small cell lung cancer as well as impaired response to chemotherapy [12]. With regard to metastasis, elevated thrombin in the bronchoalveolar fluid of osteosarcoma patients is associated with high risk of developing pulmonary metastases [13].

In other forms of tumors, expression of PAR-1, the thrombin receptor, has been shown to correlate with increased metastasis. PAR-1 over-expression has been demonstrated in high grade breast cancers [14] and melanoma cell lines isolated from metastatic lesions express higher levels of PAR-1 than those isolated from lesions that did not metastasize [15]. By contrast, immunohistochemial analysis of squamous cell carcinoma of the head and neck showed that PAR-1 expression is associated with the induction of differentiation markers and inversely correlated with lymph node metastasis [16].

Animal Models linking thrombin to cancer growth and metastasis

A few animal models to date have been used to examine linkages between thrombin activity and PAR-1 expression to metastasis. Using ATAP2 and WEDE15, antibodies which block PAR-1 cleavage and activation, Shi et al. demonstrated PAR-1 antagonism reduces human melanoma metastasis to lung secondary to tail vein injection [17]. Studies utilizing subcutaneous injection of breast cancer cells in nude and syngeneic mice demonstrated that treatment with hirudin, a thrombin inhibitor, caused a lag in initial growth, induction of central necrosis in the primary tumor, and reduced hematological spread and seeding in distant organs [18]. Using the TRAMP mouse model of prostate cancer, in which spontaneous prostate cancer develops and whose course mimics the human disease, treatment of mice with hirudin led to a reduction in spontaneous tumor growth [19].

In vitro studies and proposed mechanisms for association between thrombin and cancer pathophysiology

Cloaking Hypothesis and Dissemination

The most direct proposed linkage between thrombin activity and cancer pathophysiology is what one may deem the "Cloaking Hypothesis." This hypothesis suggests that cancer cells, especially those that express tissue factor as an initiator of coagulation, are capable of surrounding themselves with a fibrin mesh, thereby preventing immune surveillance and destruction by natural killer cells [20]. This concept is supported by the multiple demonstrations that thrombin treatment of cancer cells increases their adhesiveness to platelets [21].

In addition to its potential to initiate a fibrin "cloak," thrombin may also mediate cancer cell metastasis by enhancing extravasation at distant sites. Thrombin treatment of tumor cells has been demonstrated to increase their adhesiveness to endothelial cells and the subendothelial matrix [22]. Furthermore, at concentrations just above physiological levels, thrombin can induce barrier disruptive signaling in endothelial cells [23], thereby allowing enhanced extravasation at a potential metastatic site.

Cancer cell proliferation and motility

Multiple studies have documented the expression of functional PAR-1 in cancer cell lines and the capacity of PAR-1 activation to promote cancer cell motility including melanoma [15], prostate [24, 25], renal carcinoma [26], breast [27], and osteosarcoma [28], among others. Furthermore, downregulation of PAR-1 via siRNA targeting conversely leads to decreased motility and invasion through basement membrane extract in melanoma [15] and breast cancer cells [29]. Thrombin induced migration has been linked to a variety of signaling pathways. Kaufmann et al report that thrombin induced migration of renal carcinoma cells is dependent on activation of NFκB, PKA, MAPK, and PKC pathways [30]. PKC activation dependency was also demonstrated by Chiang et al in adenocarcinoma cells [31]. A common observation in

thrombin induced motility is induction of matrix metalloprotease (MMP) production [32, 33, 28, 34]. Using osteosarcoma cell lines, Radjabi et al have proposed a mechanism in which thrombin-mediated PAR-1 activation leads to upregulation of MMP-9 and β1 integrins, which colocalize at the cell surface to mediate invasion through a PI3K dependent pathway [28].

In addition to motility and invasion, thrombin and PAR-1 have also been shown to directly promote cancer cell proliferation. Thrombin has been demonstrated to stimulate proliferation in glioma [35], gastric [36], and colon [37] cancer cell lines. In prostate and glioblastoma cancer cells, Hu et al further demonstrated that thrombin modulates key regulators of cell cycle progression including downregulation of $p27^{Kip1}$, a universal cyclin-dependent kinase inhibitor [38]. While the precise mechanisms underlying thrombin induced cancer cell proliferation require further investigation, multiple studies have demonstrated that thrombin induced proliferation involves transactivation of the EGFR receptor [39, 36, 37, 26].

Angiogenesis

Given the well documented capacity for thrombin to promote both motility and proliferation in cancer cells, it is perhaps unsurprising that thrombin can also promote angiogenesis – a process dependent upon motility and proliferation of endothelial cells. Indeed, *in vitro*, thrombin induces tube formation in human umbilical vein endothelial cells (HUVEC) [40]. Furthermore, thrombin has also been demonstrated to activate endothelial cells to replicate and to produce angiogenic growth factors such as VEGF [41] and angiopoietin-2 [42]. With regard to the matrix remodeling that is required for the formation of new vessels, thrombin stimulates endothelial cells to produce MMP-1 and MMP-3 [43].

Activated Protein C

The role of activated protein C (APC) in coagulation is to prevent excessive thrombin generation at the site of an injury. Zymogen protein C is localized on endothelial cells by binding to the endothelial protein C receptor (EPCR) [44]. Protein C is activated by thrombin bound to the endothelium by thrombomodulin (TM) [44, 45]. In the presence of protein S [43], APC prevents further thrombin generation by proteolytically inactivating upstream coagulation factors Va and VIIIa [46, 47].

Interestingly, APC, like its activator thrombin, has been shown to activate PAR-1 in the presence of EPCR and modulate cell signaling. Despite the tight linkage between thrombin activity and APC, and the fact that they are capable of activating the same receptor, there is relatively little known about linkages between APC and cancer pathophysiology. However, there is emerging evidence that, depending on experimental context, APC can function in a fashion parallel or anti-parallel to thrombin.

Direct effects of APC on motility of cancer cells

EPCR, whose presence is required for APC activation, has been detected in breast cancer biopsies as well as multiple glioma and leukemia cell lines and can functionally activate zymogen protein C [48]. *In vitro*, Kobayashi et al demonstrated APC promotes motility of choriocarcinoma and ovarian cancer cells in the presence of the serine protease inhibitor (serpin) PAI-1 [49]. Rather than implicate signaling through PAR-1, they suggested the APC bound to PAI-1, a serine protease inhibitor that naturally inhibits uPA (reviewed below), thereby increasing the capacity of uPA to promote motility. Alternatively, it has been shown that APC promotes motility and invasion of MDA-MB-231 and MDA-MB-435 breast cancer cells by mechanisms that are dependent on EPCR and PAR-1 that involves activation of PI3K and

MAPK signals [50, 51]. Another group showed with keratinocytes [52] that APC is capable of activating pathways that promote proliferation and are anti-apoptotic and should be further investigated for its capacity to directly affect cancer cells.

Pro-angiogenic vs. barrier protective functions of APC

Much more is known with regard to the effects of APC on endothelial cells than cancer cells. However, depending on one's perspective on angiogenesis vs. barrier function, APC may act positively or negatively with regard to cancer outcomes. In this way, APC may have effects which could positively or negatively impact cancer pathophysiology in different disease stages.

Angiogenesis

Like thrombin, APC promotes motility and tube formation of endothelial cells [51, 53]. Furthermore, like thrombin, APC promotes proliferation, increased DNA synthesis, and activation of MAPK and PI3K in HUVEC and angiogenesis in mice cornea [53]. In wound healing models, APC promotes angiogenesis via increased transcription of MMP-2 in endothelial cells and VEGF in fibroblasts [54]. Beyond induction of MMP-2 and VEGF, APC may also broadly stimulate angiogenesis through its capacity to promote transcription of the pro-angiogenic cytokines monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8) in both endothelial cells and fibroblasts [55]. The studies which indicate APC promotes angiogenesis imply APC may promote cancer growth by being pro-angiogenic. However, in contrast, the capacity of APC to promote barrier function would make APC protective against tumor dissemination. There are multiple *in vivo* studies to date suggesting this function of APC.

Endothelial barrier protective activity

APC has an pro-survival effect on endothelial cells [56-58]. This is mediated by both direct signaling to endothelial cells and by modulating the production of various cytokines such as TNFα to prevent neutrophil mediated killing [59]. More mechanistically, APC has been shown to enhance HUVEC barrier integrity in a two chamber system electrical resistance model that is dependent on PAR-1 and activation of S1P pathways involved in processes such as cell proliferation, survival and migration [60]. As mentioned previously, thrombin generates barrier disruptive signaling in endothelial cells. Using HAEC, Finigan et al found APC reversed thrombin induced barrier disruption, restoring peripheral cortical actin distribution thereby restoring barrier function by a S1P dependent mechanism [61]. Supporting a role in barrier protective signaling, melanoma cells pretreated with APC exhibited decreased adhesion and transmigration through endothelial monolayers [62].

The barrier protection functions of APC are documented relative to the use of APC in treating severe sepsis; however, how this contributes to cancer pathophysiology has only recently been explored. Mice over-expressing EPCR exhibit reduced lung and liver metastasis secondary to tail vein injection [62]. In addition, treatment of mice with APC blocking antibodies decreased lung metastasis of melanoma cells, reduced extravasation as a consequence of reduced S1P activity, and reduced VE-cadherin expression in melanoma cells [63]. These *in vivo* studies examine hematological spread of injected cancer cells and strongly suggest APC would be protective against hematological metastasis. There are no *in vivo* studies to date which address the effect of APC on primary tumor growth or cancer related angiogenesis. Further studies are required to elucidate the effects of APC on primary tumor growth and local metastasis, especially in light of the similar capacities of APC and thrombin to modulate tumor cell motility and angiogenesis

In general, APC and thrombin have been shown to activate similar pathways with regard to proliferation and motility in both primary cells such as HUVEC as well as cancer cells. It is intriguing that APC and thrombin have opposite effects on endothelial cell layers given that they exert cell signaling functions via cleavage of the same receptor. Notably thrombin and APC activate PAR-1 with different efficiencies. At low levels, thrombin is barrier protective [23] and APC cleaves PAR-1 less efficiently than thrombin and requires a co-receptor. Differential signaling may therefore depend on rate of activation. It has also been demonstrated that thrombin selectively activates RhoA in endothelial cells whereas APC selectively activates Rac. This study further demonstrated APC signaling is uniquely dependent on the presence of caveolin 1 and induces PAR-1 phosphorylation but not internalization or degradation [64, 65].

Urokinase plasminogen activator

As related to hemostasis, urokinase plasminogen activator (uPA), similar to tissue plasminogen activator (tPA), catalyzes the conversion of plasminogen to plasmin. Plasmin catalyzes the degradation of fibrin, thereby mediating fibrinolysis. In the extravascular space, binding of uPA to its cognate cell surface receptor uPAR, allows for efficient activation of plasminogen in the pericellular space [66, 67]. uPA activity at the cell surface is inhibited by the cognate inhibitor of uPA, plasminogen activator inhibitor 1 (PAI-1). PAI-1 forms a 1:1 complex with uPA and renders the protease inactive, thereby inhibiting pericellular proteolysis. uPA-PAI-1 complexes are subsequently endocytosed at the cell surface by the low density lipoprotein-like receptor 1 (LRP-1). Extensive research has revealed that outside fibrinolysis, uPA and its cell receptor uPAR have broad capacity for regulating processes such as matrix remodeling, cancer cell motility and growth, as well as angiogenesis. The effects of thrombin and APC are generally

dependent on proteolysis, through PAR-1 cleavage and subsequent activation at the cell surface. uPA, by contrast, is capable of both broadly regulating matrix remodeling through protease dependent mechanisms as well as regulation of cell growth and motility through both protease-dependent and protease-independent mechanisms.

Correlations between uPA/uPAR expression and cancer development and metastasis.

Elevated uPA and or uPAR expression has been documented in diverse forms of cancer and in many cases is strongly correlated to poor disease outcomes. For example, uPA antigen is elevated in biopsies ranging from glioblastoma multiforme [68] to colorectal cancer [69]. In prostate cancer, elevated uPA antigen is found in peripheral blood [70]. In breast cancer, elevated expression of uPA and its PAI-1 [reviewed in 71] is predictive of lower rates of relapse-free survival and overall survival [72]. Elevated uPA antigen in tumor biopsies has also been shown to correlate with decreased relapse-free survival in gastric cancer [73] and shorter overall survival in colorectal cancer [74]. With regard to characterizing the aggressiveness of particular cancers, uPAR amplification is associated with invasive capacity of pancreatic adenocarcinoma [75], uPA elevation is correlated with bone metastasis in renal cancer [76], and uPA is a strong predictor of distant metastatic disease and shorter overall survival in colorectal cancer [77].

Mouse models relating uPA and cancer growth and metastasis

Mouse models utilizing a variety of tumor cell lines have demonstrated a strong etiological linkage between uPA and uPAR expression and cancer progression. In an orthotopic model of breast cancer, Kunigal et al. demonstrated that injection of uPAR targeted RNAi

constructs regressed breast tumors in mice [78]. Similarly, in a prostate cancer model, it has been demonstrated that injection of uPA and uPAR shRNA constructs into established tumors inhibits tumor growth and survival [79]. In glioblastoma models, uPA and uPAR antisense constructs can similarly reduce tumor growth lead to regression of established tumors [80, 81]. With regard to metastasis, Margheri et al demonstrated uPAshRNA constructs inhibit prostate cancer bone metastasis [82] and D-Alessio [83] et al demonstrated uPAR anti-sense treatment reduces melanoma metastasis to lung. In a finding that is promising with regard to the development of treatment strategies, treatment of mice bearing orthotopic pancreatic carcinoma cells with an anti-uPAR monoclonal antibody reduced tumor growth and retroperitoneal and liver metastasis [84]. Taking a different approach to address the contributions of tumor produced vs. host produced uPA, Gutierrez et al demonstrated diminished growth of fibrosarcoma cells in uPA null mice [85].

In vitro characterization of the effects of uPA/uPAR in cancer pathophysiology

There is a large body of research demonstrating the broad signaling capacity of uPAR. A GPI-anchored receptor, uPAR has been demonstrated to co-localize with a large number of co-receptors, including multiple integrin subunits and EGFR among others. This literature has been comprehensively reviewed [see 86, 87]. Here, we focus more narrowly on mechanisms which relate more directly to uPA.

Cancer cell motility

Put most simply, association of uPA with uPAR allows for efficient cleavage of plasminogen to plasmin in the pericellular space [66]. This initiates a proteolytic cascade that

ultimately leads to degradation of extracellular matrix (ECM) proteins surrounding a cancer cell. More specifically, plasmin can directly proteolyze ECM proteins fibronectin and vitronectin [88]. Plasmin also indirectly degrades matrix components via the activation of latent matrix metalloproteases (MMPs), including MMP-1 [89], MMP-2 [90], MMP-3 [91], MMP-11, and MMP-12 [92]. The initiation of this proteolytic cascade is inhibited by PAI-1. Collectively, as a consequence of activating a broad proteolytic cascade, uPA acts as a driver of matrix remodeling which is a key factor in cancer motility. Figure 2.1 diagrams the interplay of uPA and PAI-1 in the regulation of matrix remodeling.

uPA can also act as a direct motogenic factor independent of its capacity for plasmin generation by activating cell signaling. In MCF7 breast cancer and HT1080 fibrosarcoma cells, uPA stimulates migration via activation of the Ras, MEK, ERK signaling axis [93]. In LNCap prostate cancer cells transfected with uPAR, ligation with uPA or the amino terminal fragment of uPA (ATF), which lacks proteolytic capacity, induced motility with activation of a number of intracellular mediators of migration including FAK and p130Cas [94]. From a different perspective, it has also been demonstrated that the metastatic MCF10CA1 breast cancer cell line derived from normal epithelial MCF10A cells are more motile toward EGF as a consequence of both uPA activity and production [95].

Proliferation and Apoptosis

In addition to its capacity to regulate motility, uPA has also been demonstrated to affect both the proliferation and sensitivity to apoptosis of cancer cells themselves. uPA activates ERK signaling – a key modulator of proliferation in multiple cell types [96, 97]. Further, the ATF fragment of uPA (which lacks proteolytic capacity) stimulates proliferation in ovarian [98] and

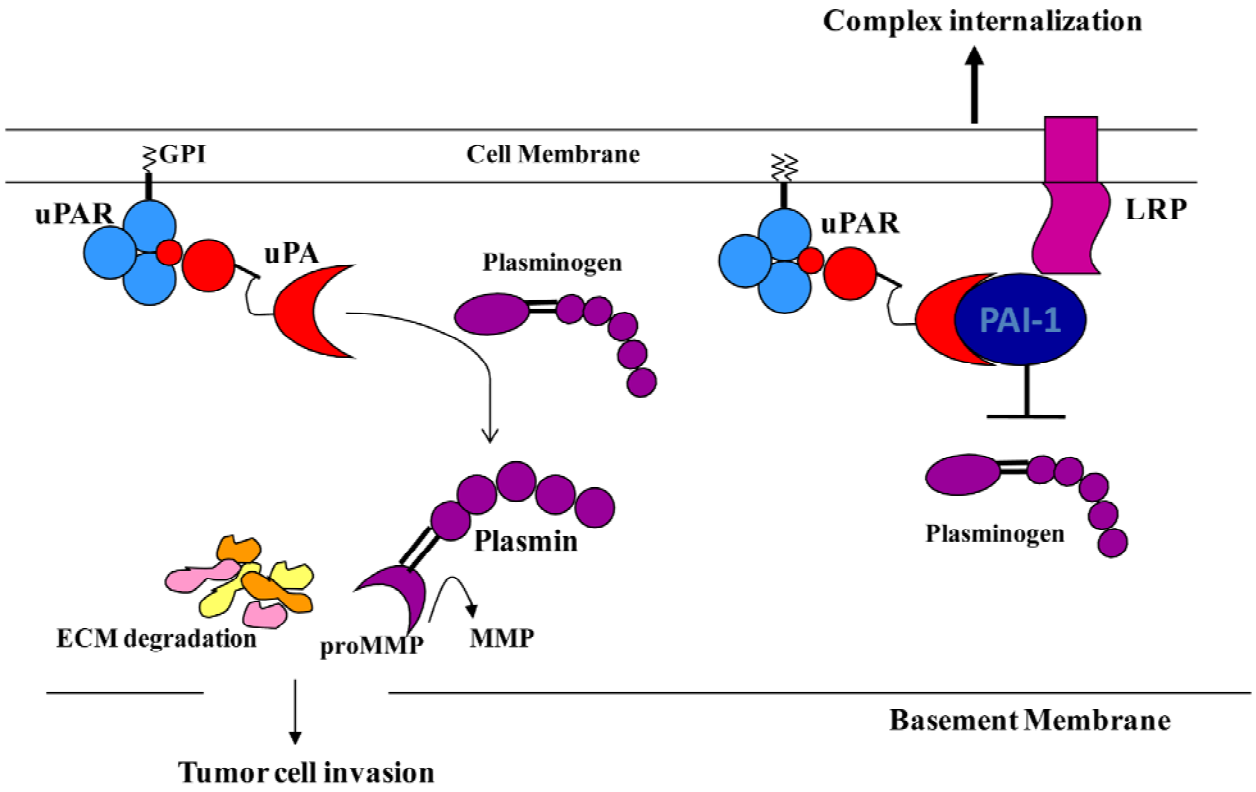

Figure 2.1. The Plasminogen Activator System at the cell surface. The catalytic activity of uPA is enhanced when bound to the cell surface by uPAR. uPA cleaves the zymogen plasminogen to its active form plasmin. Plasmin can subsequently activate MMPs in the microenvironment, thus contributing to invasion and metastasis via degradation of the ECM and basement membrane. When uPA is neutralized via covalent linkage to PAI-1, the trimeric uPAR/uPA/PAI-1 complex is bound by LRP and internalized.

osteosarcoma cell lines [99]. Other studies have suggested that uPA promotes anti-apoptotic signaling in cancer cell lines. Ma et al found that inhibition of uPA binding to uPAR in MDA-MB-231 breast cancer cells protected the cells from apoptosis, which they attributed to decreased ERK activity secondary to antibody inhibition. Glioma cells with reduced uPAR exhibit increased sensitivity to TRAIL induced apoptosis. In glioblastoma cells, Chandrasekar et al. showed that treatment of cells with uPA antisense correlated with reduced PI3K activity and survival [100].

Angiogenesis

While there is a larger body of experimental evidence linking uPA to cell motility, proliferation, and survival of cancer cells themselves, its array of biological activities has strong ties to angiogenesis. Like thrombin and APC, uPA has been shown to enhance invasive capacity of endothelial cells through matrix proteins [101] and promote tube formation [102]. Furthmore, plasmin also stimulates endothelial cell migration [103].

Both uPA and plasmin have been implicated in catalyzing the release of matrix bound growth factors or activation of pro-angiogenic factors. uPA and plasmin can both directly mediate activation of VEGF [104]. Plasmin can also catalyze the release of matrix bound VEGF [105]. In addition to VEGF, plasmin can also indirectly induce the release of ECM bound bFGF, another pro-angiogenic factor, via MMP activation [106]. Interestingly, stimulation of endothelial cells with VEGF results in the up-regulation of uPA, creating the potential for a feedback loop [107].

Conclusions

Examination of the coagulation proteases thrombin, APC, and uPA in the context of cancer physiology reveals the complex contribution of these factors to a broad array of mechanisms ranging from motility and matrix remodeling to proliferation and apoptosis. The well documented interplay of these factors in regulating hemostasis and fibrinolysis calls for continued research into how they may interact to regulate processes involved in cancer. Indeed, studies with endothelial cells reveal thrombin mediated activation of MMP-2 is dependent on the presence of TM and the generation of APC, suggesting activities once thought mediated by thrombin or APC alone may require both proteases [108]. Furthermore, it has been recently shown that treatment of mouse breast cancer cells with thrombin can induce bolus release of uPA from intracellular stores [109]. Moving forward, it will be important to determine if coordinate activities of thrombin, APC, and uPA contribute to cancer growth and metastasis.

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Chapter III

Activated Protein C Enhances Cell Motility of Endothelial Cells and MDA-MB-231 Breast Cancer Cells by Intracellular Signal Transduction

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M.W. Gramling, L.M. Beaulieu, F.C. Church. Activated protein C enhances cell motility of endothelial cells and MDA-MB-231 breast cancer cells by intracellular signal transduction, Exp Cell Res (2010) 316 314-328.

Abstract

Activated protein C (APC), an anticoagulant serine protease, has been shown to have non-hemostatic functions related to inflammation, cell survival, and cell migration. In this study we investigate the mechanism by which APC promotes angiogenesis and breast cancer invasion using *ex vivo* and *in vitro* methods. When proteolytically active, APC promotes cell motility/invasion and tube formation of endothelial cells. *Ex vivo* aortic ring assays verify the role of APC in promoting angiogenesis, which was determined to be dependent on EGFR and MMP activation. Given the capacity of APC to promote angiogenesis and the importance of this process in cancer pathology, we investigated whether the mechanisms by which APC promotes angiogenesis can also promote motility and invasion in the MDA-MB-231 breast cancer cell line. Our results indicate that, extracellularly, APC engages EPCR, PAR-1, and EGFR in order to increase the invasiveness of MDA-MB-231 breast cancer cells. APC activation of matrix metalloprotease (MMP) -2 and/or -9 is necessary but not sufficient to increase invasion, and APC does not utilize the endogenous plasminogen activation system to increase invasion. Intracellularly, APC activates ERK, Akt, and NFκB, but not the JNK pathway to promote breast cancer migration. Similar to the hemostatic protease thrombin, APC has the ability to enhance both endothelial cell motility/angiogenesis and breast cancer cell migration.

Introduction

Activated protein C (APC), a liver-derived serine protease [1] has known functions *in vivo* as an anticoagulant. Zymogen protein C (PC) is localized to the endothelium by binding to endothelial cell protein C receptor (EPCR) [2]. Thrombin bound to the endothelial cell surface by thrombomodulin (TM) [2,3], cleaves PC into its active form. Along the periphery of the clot, APC proteolytically inactivates factors Va and VIIIa [4,5] in the presence of protein S (PS) [6,7]. In humans, severe thrombophilia occurs with deficiencies in PC or PS and with a mutation in factor Va that prevents its inactivation by APC, known as Factor V Leiden [8]. Recently, in the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) Study, patients diagnosed with sepsis and acute organ dysfunction were treated with recombinant human APC, resulting in a mortality reduction of 19.4% [9].

APC has a role in regulating migration of various cell types. Kobayashi, *et al*. [10] originally reported APC promotes invasion of ovarian cancer and choriocarcinoma cells only in the presence of the serine protease inhibitor (serpin) plasminogen activator inhibitor-1 (PAI-1). They suggest APC forms a stable complex with PAI-1, thereby removing a potent inhibitor of urokinase plasminogen activator (uPA) from the system [10]. uPA directly activates matrix metalloprotease (MMP)-2 and uPA can also regulate MMP-9 expression [11-13], both of which are zinc-dependent proteases that degrade the ECM. Plasmin, which is activated directly by uPA, can also activate both MMP-9 [14] and MMP-2 [15]. Previously, APC has been shown to activate MMP-2 from an intermediate to a fully active protease [16-19]. *In vitro* cell culture assays with keratinocytes have shown that APC promotes cell migration by increasing both the expression and activation of MMP-2 [19]. *In vivo*, APC promotes angiogenesis and wound healing by reducing inflammation, increasing VEGF expression, and increasing MMP-2 [17].

Beyond its potential for manipulating the activation of MMP's, APC has also been implicated in intracellular signal transduction pathways, which alter proliferation and migration. APC has been shown to increase endothelial cell proliferation and *in vivo* angiogenesis in a concentration dependent manner through activation of the mitogen activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K), and endothelial nitric oxide synthase (eNOS) pathways [20]. It has also been shown that APC, in the presence of caveolin-1, activates Rac1 through PAR-1 to promote protective barrier signaling in immortalized HUVEC [21]. There is relatively limited data on APC induced signal transduction in cell types outside of endothelial cells. However, it has been shown that in epidermal keratinocytes, APC can stimulate the MAPK pathway via transactivation of the epidermal growth factor receptor (EGFR) [22].

Our primary objectives in this study were to (1) confirm the role of APC in promoting angiogenesis using both *in vitro* and *ex vivo* models; (2) explore the role of MMP and EGFR activation in APC stimulated angiogenesis; and (3) examine the mechanism that accounts for our previous finding that APC can promote motility in MDA-MB-231 breast cancer cells [23]. The results described here provide evidence that MMP and EGFR activation are necessary for APC induced angiogenesis. Furthermore, the results indicate that APC promotes breast cancer cell motility and invasion through pathways similar to those previously reported for endothelial cells and keratinocytes, but different from the mechanism reported for other cancer cells. Specifically, this mechanism involves binding to EPCR, activation of both PAR-1 and EGFR to promote intracellular signaling through MAPK and PI3K pathways, and extracellular interactions with MMP-2 and -9 to support ECM degradation.

Material and Methods

Cell Culture. Human umbilical vein endothelial cells (HUVEC), obtained from Cambrex, were grown according to manufacturer's specifications. Cells were grown in endothelial cell basal media with 2% fetal bovine serum (FBS), bovine brain extract (BBE) with heparin, GA-1000, human epidermal growth factor (hEGF), and hydrocortisone (Cambrex). Assays were performed using the MDA-MB-231 breast cancer cell line obtained from the University of North Carolina – Chapel Hill Tissue Culture Facility. These cells were maintained in Minimum Essential Media (MEM; Gibco) with 10% FBS (Sigma), 1% sodium pyruvate (Gibco), and 1% antibiotic/antimycotic (Gibco). All cells were cultured in an incubator at 37^{o}C, 5% CO_2.

Immunofluorescence. In the murine *ex vivo* aortic ring assays, after 5 days in culture, aortic sections were fixed in 4% paraformaldehyde in 1X PBS for 30 minutes on ice. After 2 washes in 1X PBS, the sections were permeabilized with 0.5% Triton X-100 in 1X PBS for 15 minutes on ice. To block any reactive aldehyde groups, the sections were treated with 0.2 M glycine for 20 minutes on ice and washed. Each section was blocked overnight in 10% goat serum in 1X PBS, 1% BSA at 4^{o}C. The following day each section was washed in 1X PBS and then treated with a rabbit polyclonal anti-von Willebrand factor (vWF) antibody (1:50 in 1X PBS, 1% BSA; Dako) or normal rabbit serum, overnight at 4^{o}C. The next day, each aortic section was washed 5 times with 1X PBS, 1% BSA, then allowed to sit in this wash solution overnight at 4^{o}C. Each section was treated with goat F(ab´)$_2$ anti-rabbit IgG conjugated to Lissamine Rhodamine-B (1:50 in 1X PBS, 1% BSA; BioSource International), covered, and stored overnight at 4^{o}C. Each aortic section was washed 5 times with 1X PBS and stored in 1X PBS,

covered at 4^{o}C, until photographs could be taken with the Olympus IMT2 Inverted Flourescence Microscopy using a TRITC filter.

For experiments with MDA-MB-231 cells, cells grown to form a confluent monolayer in 2-chambered slides (Lab Tek II by Nunc) were fixed and stained as previously described [23] without permeabilization. Cells were blocked in 10% goat serum in 1X PBS, 1% BSA for 30 minutes and then treated with mouse EGFR antibody (Upstate) or serum mouse IgG (5 µg/mL; Sigma) for 1 h. After multiple washes in 1X PBS, 1% BSA, cells were treated with sheep anti-mouse IgG F(ab´)$_2$ fragment-R-phycoerythrin antibody (1:20; in 1X PBS, 1% BSA) for 1 hour. Cells were then washed multiple times in 1X PBS and stored in 1X PBS at 4^{o}C. Photographs were taken with an Olympus DP70 Microscope Digital Camera with DP70-BSW Software using an Olympus BX51WI fluorescent microscope with a TRITC filter. Photographs were taken at a 200X magnification with the same exposure time.

Tube Formation Assay. In a 24-well plate, wells were coated with Growth Factor-Reduced Matrigel (BD Biosciences) and incubated for 1 h at 37^{o}C. 80,000 HUVEC/well were plated with increasing concentrations of APC (0-10 µg/mL; Xigris®; Eli Lilly and Co.), PC (10 µg/mL Hematologic Technology), APC-DEGR (10 µg/mL Hematologic Technology), or VEGF (100 ng/mL R&D Systems) and incubated at 37^{o}C for 18 h. Photographs were taken at 6, 12, and 18 h using Adobe Photoshop and a Kodak DC290 Digital Camera mounted on an inverted microscope. The number of tube-like structures (defined at a structure no more than 3 cells wide with junction points on each end) were counted in 4-100X views.

Murine Ex Vivo Aortic Ring Assay. Wildtype C57BL/6 (Charles River) mice were maintained by the Veterinary Staff of the University of North Carolina at Chapel Hill

Department of Laboratory Animal Medicine and all mouse protocols were reviewed and approved by the University's Institutional Animal Care and Use Committee (IACUC).

Based on previous work [24], male and female mice, at varying ages, were anesthetized with 1.25% tribromethanol (0.2 mL/10g) and monitored by toe reflex response and respiratory rate. Once properly anesthetized, an incision was made midline and the organs were removed to expose the aorta from the heart to the renal arterial branch point. The aorta was dissected from the heart to the split for the renal arteries. The dissected aorta were placed in a 100 mm dish containing Dulbecco's Modified Eagle Media (DMEM; Gibco) with 1% antibiotic/antimycotic (Gibco) and cleaned of connective tissue and fat. Cleaned aortas were then sectioned into 1-2 mm pieces and placed into a new 100 mm dish containing DMEM with 1% antibiotic/antimycotic and allowed to incubate for approximately 1 h at 37^{o}C, 5% CO_2 until implantation. In a 48-well plate, 200 µL of Growth Factor-Reduced Matrigel was plated in each well and placed in an incubator at 37^{o}C, 5% CO_2 for 10 minutes. One aortic section per well was placed on top of the gelatinized Matrigel, covered with an additional 200 µL of Growth Factor-Reduced Matrigel, and incubated for 10 minutes. 200 µL of endothelial basal media with 2% FBS, BBE with heparin, GA-1000, hEGF, and hydrocortisone (Cambrex) was added to each well with either no additional treatment (negative control), 100 ng/mL VEGF (positive control), or increasing concentrations of APC (0-10 µg/mL). To eliminate any effects of thrombin on sprout formation, 50 nM of hirudin was added to the aortic sections with or without APC (10 µg/mL). To determine if the active form of APC was needed to increase sprout formation, aortic sections were also treated with PC (10 µg/mL) with or without hirudin (50 nM) or APC-DEGR (10 µg/mL). In studies looking at EGFR, PI3K, and MMPs, pharmacological inhibitors, AG 1478 (10 µM;Biomol) [25], LY 294002 (10 µg/mL; Biomol) [26], and GM 6001 (10 µg/mL;Biomol)

[27], respectively, were added to the aortic sections 30 minutes prior to the addition of APC (10 μg/mL). Every 24 h for 5 days, media from each well was removed and replaced with fresh conditioned media. On days 3-5, the number of sprouts extending form the periphery of the aortic sections was counted and digital photographs were taken using Adobe Photoshop and a Kodak DC290 Digital Camera mounted on an inverted microscope.

Western Blots. 25 μg total protein from cell lysates created in complete RIPA buffer were run on 12% SDS-PAGE gels and transferred onto PVDF (Millipore). Cell lysates were probed for P-ERK1/2 (Santa Cruz Biotechnology), total ERK2 (Santa Cruz Biotechnology), P-Akt (Cell Signaling), total Akt (Cell Signaling), P-c JUN (Cell Signaling), total c JUN (Cell Signaling), IκB-α (Cell Signaling), MMP-2/9 (Chemicon), tubulin (Sigma), and actin (Santa Cruz Biotechnology).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Assays Using Epidermal Growth Factor. MDA-MB-231 cell viability was measured using a MTT assay. 10,000 cells/well were plated in a 96-well plate, incubated overnight at 37^{o}C, 5% CO_2. Cells were treated with SFM containing 1% sodium pyruvate, 1% antibiotic/antimycotic with mouse EGFR antibody or mouse serum IgG for 15 min followed by the addition of recombinant hEGF (1nM final concentration; Invitrogen). After a 48 h incubation, cells were treated with SFM with 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (0.5 mg/mL; Sigma) for 2 h. DMSO was added to each well to dissolve the formazan crystals formed by living cells for 15 min at 37^{o}C, 5% CO_2. The absorbance of each well was read at 560 nm.

Transwell Invasion and Chemotaxis Assays. Migration was evaluated as previously described using the transwell invasion and chemotaxis assays [23, 28, 29]. For endothelial cells, the chemotactic agent, 1 ng/mL VEGF (R&D Systems) in culture media was added to the well.

50,000 cells/insert were treated with increasing concentrations of APC (0-10 µg/mL; Xigris®; Eli Lilly and Co.) in culture media and added to the insert. Endothelial cell invasion and chemtoxis assays were both incubated for 24 hr. MDA-MB-231 cells, also plated at 50,000 cells/insert, migrated towards 10% FBS (the chemotactic agent) containing media. Chemotaxis assays for the breast cancer cells were incubated for 12 h, while the invasion assays were incubated for 24 h. All experiments were done at 37^{o}C, 5% CO_2. Blocking antibodies used to study receptors potentially bound by APC were plated with the cells and allowed to incubate at room temperature for 15 min prior to the addition of APC. These antibodies include mouse EPCR antibody (JNK 1494; provided by Dr. Charles T. Esmon, OMRF, Oklahoma, OK), mouse PAR-1 antibodies (atap2 and wede15; Zymed and Immunotech, respectively), mouse EGFR antibody (Upstate), and mouse serum IgG (Sigma). Blocking antibodies used to study the role of the plasminogen activation system, including goat PAI-1 antibody (American Diagnostica), mouse uPA antibody (American Diagnostica), and serum mouse or goat IgG (Sigma), were plated with the cells and allowed to incubate at room temperature for 1 h prior to the addition of APC. Amiloride, an inhibitor of uPA, was also preincubated for 1h with the cells. Pharmacological inhibitors that block specific signaling pathways were plated with the cells and allowed to preincubate at room temperature for 1 h. These compounds include PD 98059 (Biomol), LY 294002 (Biomol), and SP 600125 (Biomol). Finally, inhibitors for MMPs, GM 6001 (Biomol) and SB-3CT (Biomol) were plated with the cells and preincubated for 15 min prior to the addition of APC. To determine if selective inhibitors of MMPs were affecting APC activity levels, conditioned media from the transwell assays were tested for APC activity using an APC-specific chromogenic substrate (final concentration of 0.15 mM; Centerchem) as previously described [23].

Quantitative PCR. Serum starved MDA-MB-231 cells at ~85% confluence were serum starved for 24 h, then treated with SFM containing increasing concentrations of APC (0, 100 ng/ml, 1 μg/ml, 10 μg/ml). After isolation through Trizol (Invitrogen), RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using SYBR Green Dye (Applied Biosystems) and the following protocol: 5 minute initial denaturation step at 95°C, followed by 40 cycles of 95°C for 20-s, 55°C for 1 min, and 68°C for 30-s. Using β-actin as a loading control, results were interpreted by the relative quantity method (ΔΔCt) as described [30]. Primer sequences were: MMP-2 sense 5'-ATAACCTGGATGCCGTCGT-3', MMP-2 anti-sense 5'-AGGCACCCTTGAAGAAGTAGC-3', MMP-9 sense 5'-CGGTGATTGACGACGCCTTT-3', MMP-9 anti-sense 5'-ACCAAACTGGATGACGATGTCTG-3', β-actin sense 5'-ATCATGTTTGAGACCTTCAA-3', and β-actin anti-sense 5'-CATCTCTTGCTCGAAGTCCA-3'.

Statistical Analysis. For each transwell experiment, conditions were done in duplicate or triplicate and averaged. Averages of each condition were compared to No Treatment, APC alone, or blocking antibody/inhibitor alone. Experiments were repeated as indicated in the figure legends and averaged together. Results show the percentages compared to No Treatment, which is set to 100%. Statistical analysis was performed on each comparison – to No Treatment, APC, or blocking antibody/inhibitor – using a one sample T-test with a normal distribution, a theoretical mean of 100, and significance of $p < 0.05$. For quantitative real time PCR, ΔΔCt values were averaged from three independent experiments and compared to a theoretical mean of 1, also using a one sample T-test, $p<0.05$.

Results

APC Promotes Endothelial Cell Motility and Angiogenesis. Given the previous reports *in vivo* that APC may directly promote angiogenesis, we were interested in closely examining this phenomenon using an *in vitro* and *ex vivo* models in parallel. For the *in vitro* model, HUVEC were treated with increasing concentrations of APC in the transwell chemotaxis and invasion assays, with VEGF (1 ng/mL) as the chemotactic agent. As seen in Figure 3.1A, APC optimally promoted invasion at 0.1 µg/mL and chemotaxis at 0.5 µg/mL. Notably, this APC-promoted increase in invasion and chemotaxis occurs at concentrations that are below the physiological level of PC found in plasma (~4 µg/mL). The highest concentration of APC (10 µg/mL) used in this assay actually reduced both invasion and chemotaxis of HUVEC compared to No Treatment. These results suggest that, in these experimental conditions, HUVEC invasion and chemotaxis are increased at a physiologically relevant concentration of APC, but are inhibited at much higher levels.

HUVEC plated onto growth factor-reduced Matrigel will form a network of tube-like structures, defined as 1-3 cells wide with junction points at both ends (as seen in Figure 3.1B, No Treatment). Upon the addition of a pro-angiogenic factor, VEGF, HUVEC will form an increased number of stable tube-like structures (Figure 3.1B, VEGF treatment). As with the transwell assays, treatment with increasing concentrations of APC (0-10 µg/mL) increases the formation of tube-like structures by 130% at an optimal concentration (1 µg/mL) and at an optimal timepoint (12 h) (Figure 3.1C), similar to the effects of VEGF (data not shown).

To determine if the effects of APC on tube formation are due to its proteolytic activity, HUVEC were also treated with 2 inactive forms of APC, the zymogen PC (1 µg/mL) and the chemically inactive protease, APC-DEGR (1 µg/mL). At 12 h, only the active protease is able to

significantly increase the formation of tube-like structures by 135% compared to No Treatment (Figure 3.1B and C). These results suggest that APC must proteolytically cleave another protease or receptors in order to increase the formation of tube-like structures.

For the *ex vivo* model we utilized the murine aortic ring assay. In this assay, murine aortic sections produce sprouts composed of endothelial cells along the periphery of the vessel that will lengthen and branch over time. Upon treatment with a pro-angiogenic factor, such as VEGF, there will be an increase in the number of endothelial cell sprouts, the length of the sprouts, and their ability to survive in culture. As seen in Figure 3.1D, increasing concentrations of APC (0-10 μg/mL) increased sprout formation around each section compared to No Treatment. Only the highest concentration of APC (10 μg/mL) was able to significantly increase the amount (300-400% compared to No Treatment) of sprouts earlier (Day 3) than VEGF (Day 4). Immunofluorescence staining for vWF, an endothelial cell-specific marker, was done to verify that the cells responding to APC treatment were in fact endothelial cells. As seen in Figure 3.1E, both VEGF and APC were able to increase sprout formation of vWF-positive endothelial cells. Therefore, APC increased the formation of endothelial cell sprouts on murine aortic rings similar to the pro-angiogenic factor, VEGF.

To determine if the effects shown with APC are specific for this protease and not another serine protease, thrombin, aortic sections were also treated with hirudin (50 nM), a potent and specific inhibitor of thrombin that has been previously shown to inhibit thrombin-promoted cell migration [23]. As seen in Figure 3.1F, at day 4, APC increases sprout formation 300-400% compared to No Treatment, in the presence and absence of hirudin. The effects of hirudin alone are not significant. Further, PC, PC/hirudin, and APC-DEGR did not significantly increase

sprout formation over No Treatment (Figure 3.1F). Therefore, APC promotes endothelial sprout formation, independent of any thrombin present in this assay.

APC promotes angiogenesis through MMP and EGFR activation. Having shown APC promotes angiogenesis in both our *in vitro* and *ex vivo* models, we examined whether the response is due to activation of MMP and EGFR, as it has been reported both are involved in APC mediated motility and proliferation in keratinocytes [19]. To determine if MMPs are important in APC-promoted angiogenesis, the general MMP inhibitor, GM 6001, was used in the *ex vivo* murine aortic ring assay. Compared to No Treatment, there was no effect of GM 6001 on sprout formation at day 4 (Figure 3.2A). APC alone increases sprout formation 400% compared to No Treatment. When the aortic sections were treated with APC and GM 6001, there is no longer an increase over No Treatment as seen with APC alone, suggesting GM 6001 blocks the effect of APC on endothelial cell sprout formation (Figure 3.2A). Sprouts that form around the periphery of the aortic section treated with GM 6001 and APC are neither as numerous, nor as long as the ones that form with APC treatment alone. Taken together, these results imply that APC promotes angiogenesis through MMPs.

To determine if EGFR activation is involved in APC promoted angiogenesis, murine aortic rings were treated with a chemical inhibitor, AG 1478, that blocks ligand binding to EGFR. As seen in Figure 3.2B, at day 4, AG 1478 alone reduces the formation of sprouts because of the importance of this receptor in angiogenesis. If EGFR is not important to the mechanism utilized by APC to increase endothelial cell sprout formation, then APC treatment with the inhibitor would be expected to have increased sprout formation relative to inhibitor treatment alone. However, in the presence of AG 1478, APC is unable to increase endothelial cell sprout

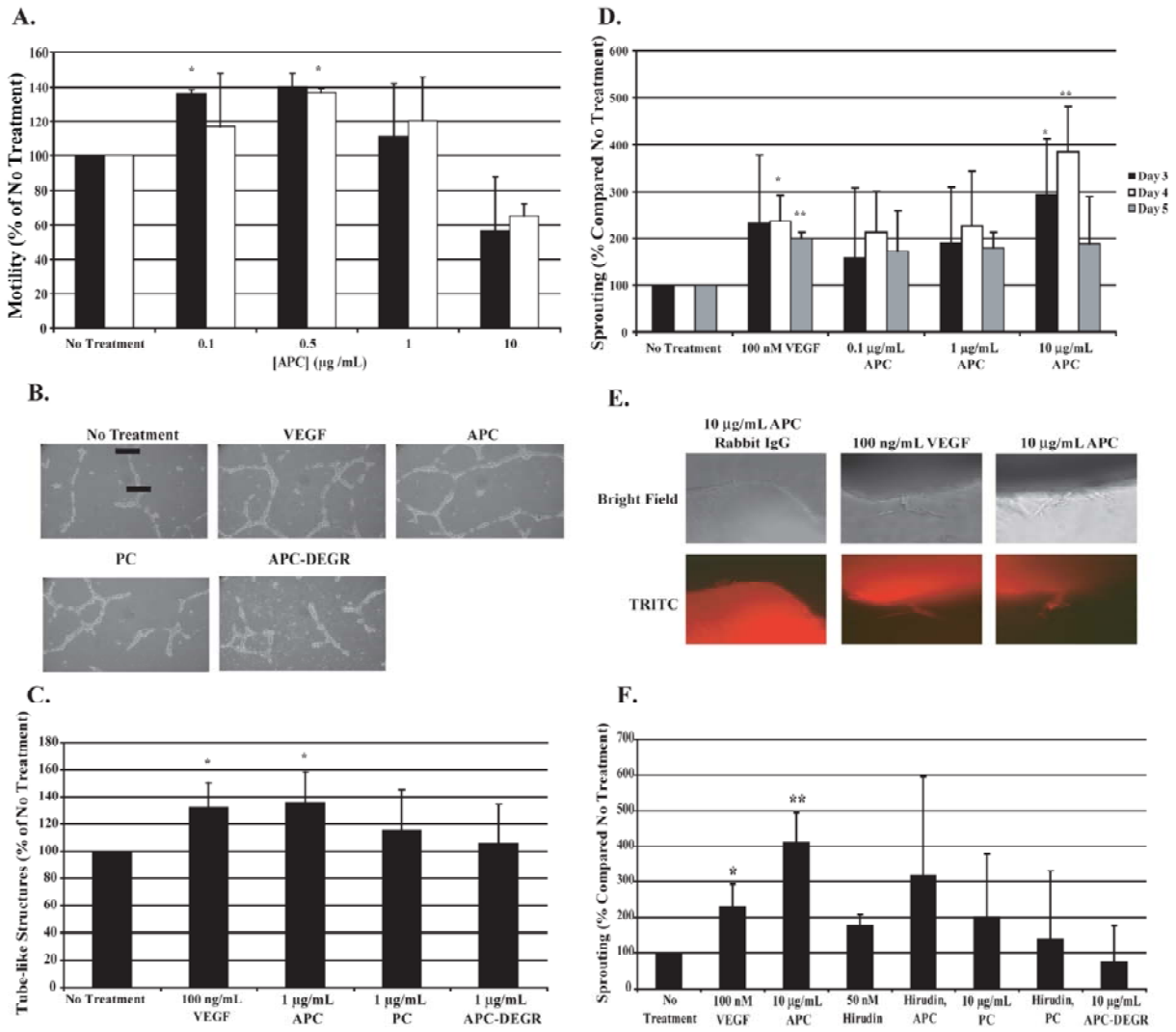

Figure 3.1: APC promotes HUVEC invasion, chemotaxis, tube formation and murine aortic ring sprouting. (A) HUVEC were treated with increasing concentrations of APC (0-10 μg/mL) for 24 h, using 1 ng/mL VEGF as the chemotactic agent. Black bars are transwell invasion results and the white bars are chemotaxis results. (B) Representative photographs of HUVEC plated onto Growth Factor-Reduced Matrigel and treated with APC (1 μg/mL), PC (1 μg/mL), APC-DEGR (1 μg/mL), or VEGF (100 ng/mL; positive control) for 12 h. (C) The number of tube-like structures (defined as 3 cells wide or less with junction points on both ends) were quantified at the 12 h time point. All graphs represent the average of 3 separate experiments.
* $p<0.05$ compared to No Treatment. (D) Murine aortic rings were treated with increasing concentrations of APC (1-10 μg/mL) or VEGF (100 nM) for 5 days. On days 3-5, the number of sprouts projecting from the periphery of the aortic sections was counted. Graph represents the average of 4 separate experiments. * $p<0.05$, ** $p<0.01$ compared to No Treatment. (E) Representative immunofluorescence staining of aortic rings for vWF. (F) Murine aortic rings were treated with APC (10 μg/mL) ± hirudin (50 nM), PC (10 μg/mL) ± hirudin (50 nM), APC-DEGR (10 μg/mL), or VEGF (100 nM) for 5 days. Graph represents the average of 2-3 separate experiments from data collected on day 4. * $p<0.05$, ** $p<0.01$ compared to No Treatment.

formation relative to the number of sprouts with AG 1478 alone. Therefore, EGFR is involved in the mechanism utilized by APC to increase angiogenesis.

APC Promotes MDA-MB-231 Breast Cancer Invasion through EPCR and PAR-1. It has been well documented that APC's ability to promote angiogenesis and alter signaling is dependent on binding to cell surface receptors EPCR and PAR-1. Previously, we showed that APC binds to both EPCR and PAR-1 to increase chemotaxis of the MDA-MB-231 breast cancer cell line [23]. To further examine the role of these receptors in the overall motility of breast cancer cells, we studied the effects of blocking EPCR and PAR-1 in the transwell invasion assay. Addition of a blocking antibody to EPCR abrogated the effects of APC on invasion compared to APC treatment alone (Figure 3.3A). The PAR-1 blocking antibodies wede15 and atap2 also hindered the effects of APC, reducing the level of invasion back to No Treatment (Figure 3.3B).

APC Promotes MDA-MB-231 Breast Cancer Chemotaxis through MAPK and PI3K/Akt Activation. Based on previous work that suggested APC is capable of activating the MAPK and PI3K/Akt pathways in other cell types, we investigated whether APC can activate these pathways in the MDA-MB-231 cells and the impact of these pathways on invasion. First, cells were treated with increasing concentrations of APC. As shown in Figure 3.4A, at all concentrations of APC used, APC activated PI3K as evidenced by increased phosphorylation of Akt. Furthermore, MAPK activity increased in a dose dependent response to APC as evidence by increased phosphorylation of ERK1/2. We further studied the MDA-MB-231 cells using the transwell assays to determine if PI3K and MAPK activity had an impact on APC-promoted cell chemotaxis (Figure 3.4B). LY 294002 was used to inhibit PI3K activity and reduce phosphorylation of Akt. LY 294002 completely reduced P-Akt levels (Figure 3.4C) at 50 μM without affecting cell viability. In the transwell chemotaxis assay, LY 294002 caused a loss of

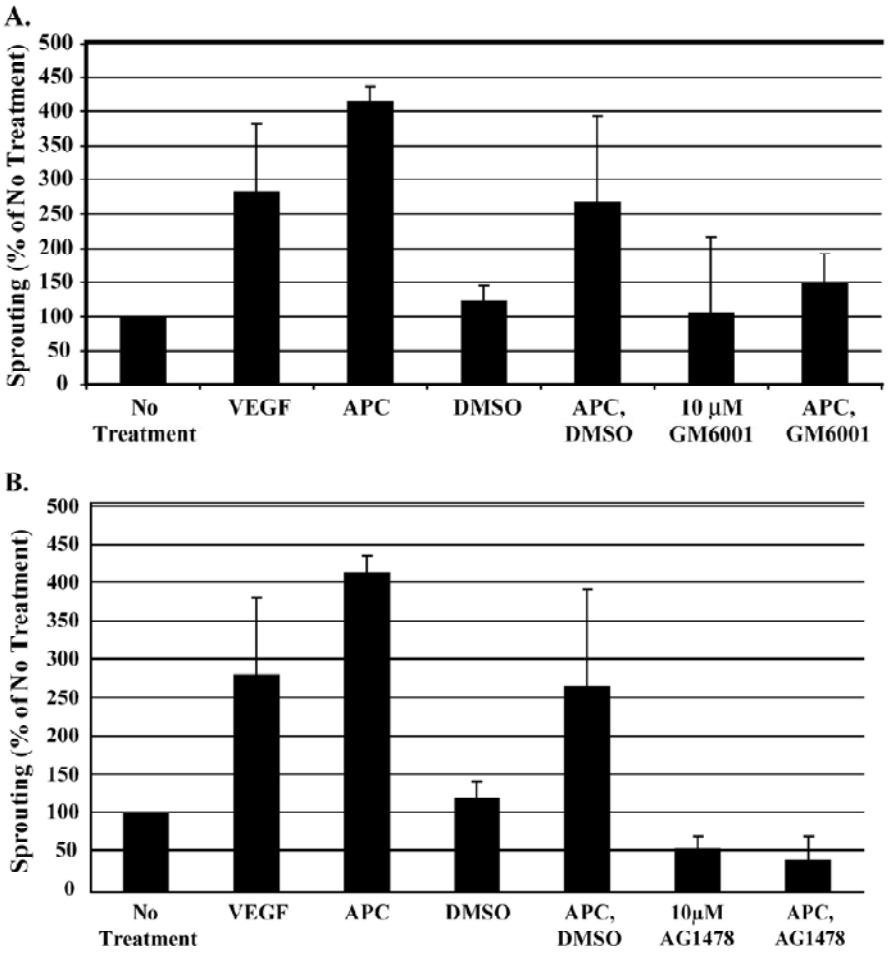

Figure 3.2. APC promotes sprout formation of murine aortic rings through MMPs and EGFR. Murine aortic rings were pretreated on day 1 with 10 µM of GM 6001 (A) or AG 1478 (B) for 30 minutes prior to the addition of APC (10 µg/mL). Graphs represent the average of 2 separate experiments from data collected on day 4.

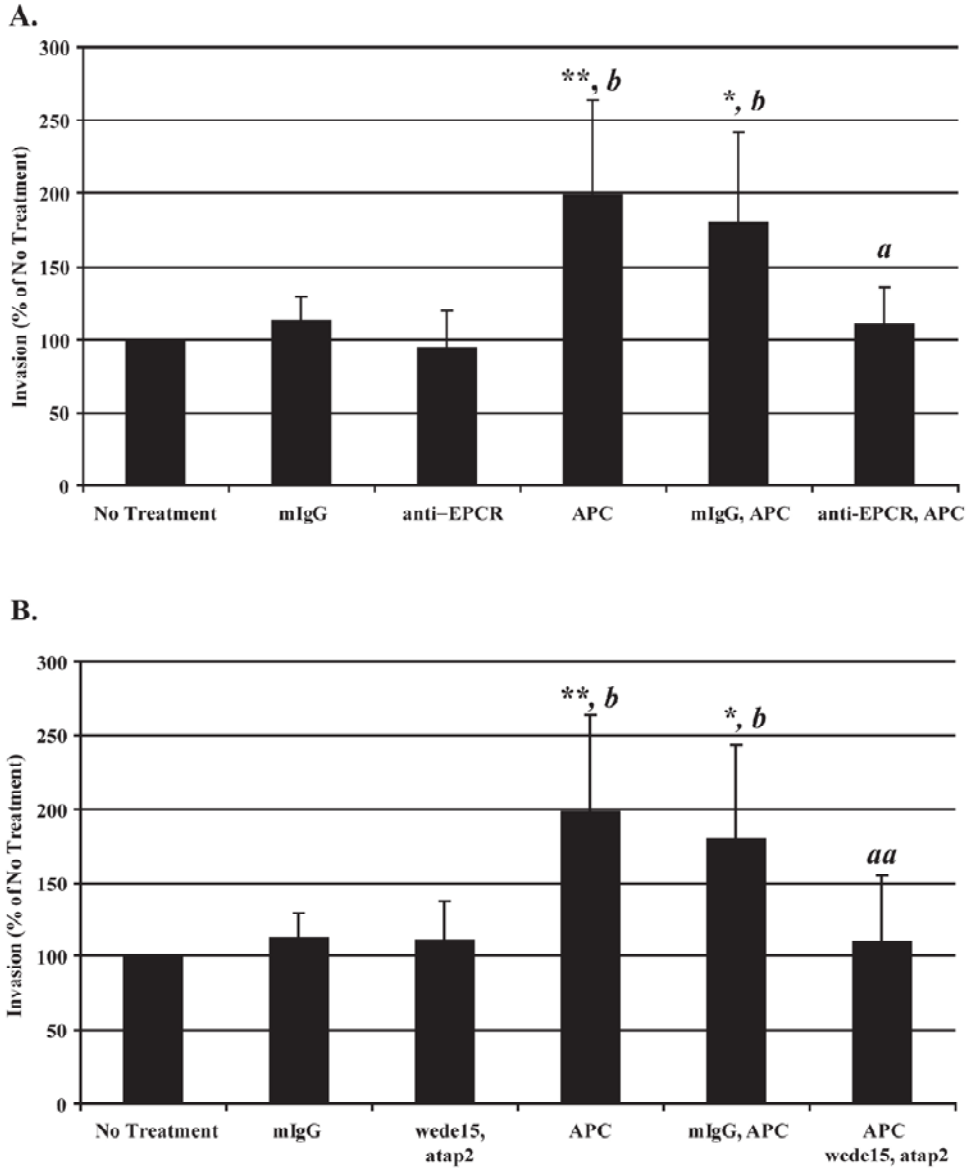

Figure 3.3. APC promotes invasion of the MDA-MB-231 cells by binding to EPCR and PAR-1. (A) Cells were pretreated with 4 μg/mL EPCR antibody (JNK 1494) or control mouse serum IgG for 15 minutes prior to the addition of 10 μg/mL APC. (B) In a separate experiment, cells were pretreated with 10 μg/mL atap 2 and 20 μg/mL wede15 (PAR-1 blocking antibodies) or control mouse serum IgG for 15 minutes prior to the addition of 10 μg/mL APC. Cells invaded and migrated toward 10% FBS containing media for 24 h. The graphs represent the average of 8 separate experiments for both (A) and (B); * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p< 0.05$, *aa* $p<0.01$ compared to APC treatment; *b* $p<0.05$ compared to mIgG treatment.

chemotaxis (Figure 3.4B), as expected since this is an important signaling pathway for cell migration. If this pathway was not activated by APC, we would expect that combined APC and LY 294002 treatment would be increased over LY 294002 alone. However, APC and LY 294002 treatment was the same as LY 294002 treatment alone (Figure 3.4B), indicating that APC promotes cell migration through a PI3K dependent pathway.

The PD 98059 compound was used to inhibit MEK1/2 phosphorylation of ERK1/2 (Figure 3.4E). The 20 μM concentration was used in the transwell assay as it did not inhibit cell survival as seen with higher concentrations. Because the MAPK pathway is an important signaling pathway for cell migration, treatment with the PD 98059 compound alone reduced chemotaxis (Figure 3.4D). Combined APC and PD 98059 treatment was reduced to the same level as PD 98059 treatment alone, suggesting that APC promoted cell migration also depends on MAPK activity.

Notably, chemotaxis assays were also performed using a c JUN inhibitor, SP 600125, which inhibits JNK phosphorylation. Despite the documented links between the JNK pathway and EGFR activation [31] and PAR-1 activation [32], treatment with SP 600125 did not inhibit APC induced chemotaxis (data not shown), suggesting that the JNK pathway is not involved in APC mediated motility.

APC Promotes MDA-MB-231 Breast Cancer Motility through EGFR Activation. Having established that EGFR activation is involved in the ability of APC to promote chemotaxis and angiogenesis in endothelial cells, we investigated whether EGFR activation is also involved in the chemotaxis and invasion of MDA-MB-231 breast cancer cells in response to APC. We verified through immunofluorescence the expression of EGFR on the cell surface (Figure 3.5A). To confirm this receptor was functional, MDA-MB-231 cells were treated with EGF and cellular

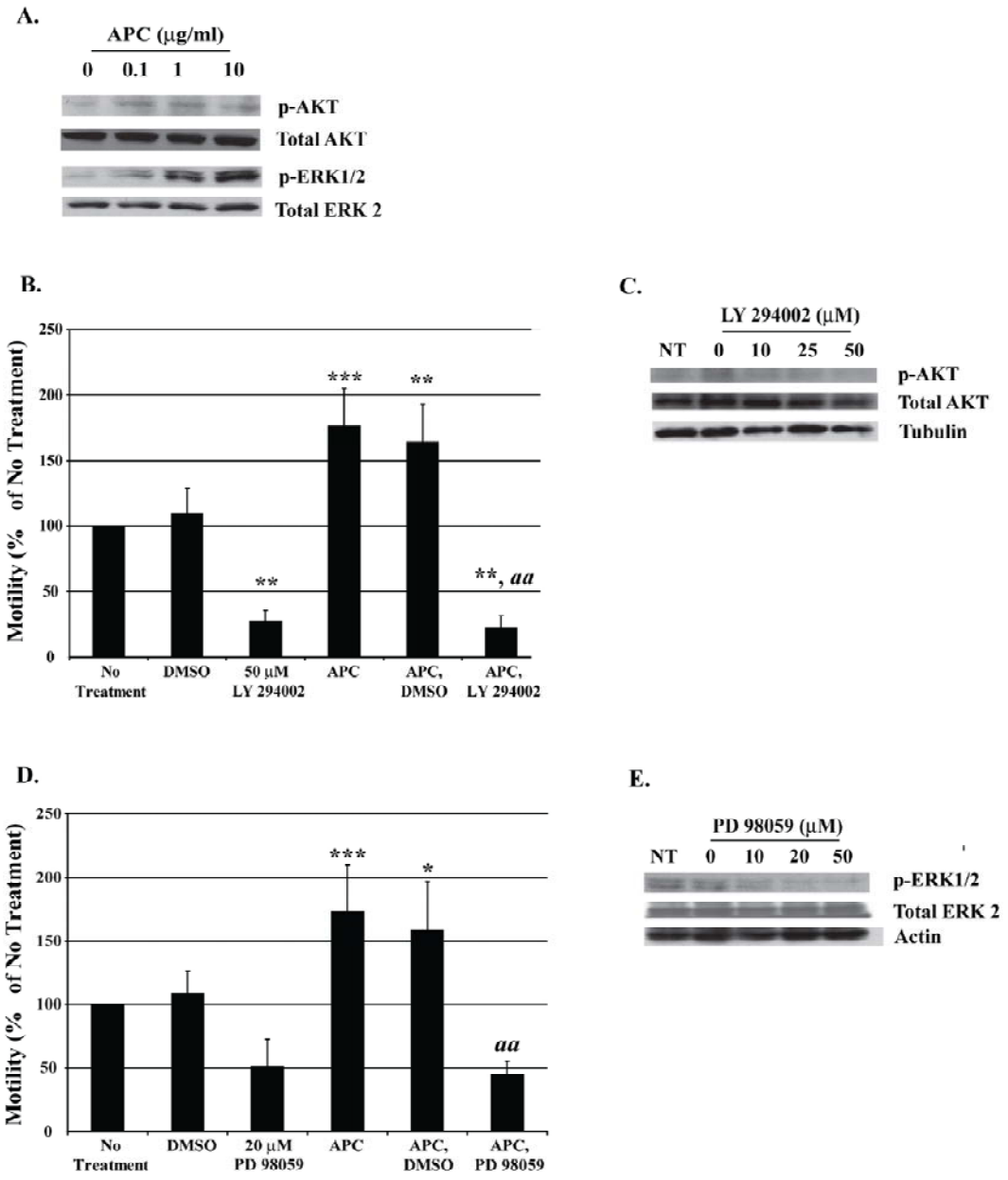

Figure 3.4. APC promotes chemotaxis of MDA-MB-231 cells by activating both the PI3K and MAPK pathways. (A) Cells were treated with increasing concentrations of APC (0, 0.1, 1, and 10μg/ml) for 24 h and cell lysates were immunoblotted for phosphorylated and total Akt, as well as phophorylated ERK1/2 and total ERK2. Additionally, cells were treated with increasing concentrations of (C) LY294002 (0-50 μM) and (E) PD98059 (0-50 μM), or (C and E) DMSO for 12 h to determine the optimal concentration of each inhibitor to be used in the transwell assay. Cells lysates were probed for phosphorylated and total Akt (C), phosphorylated ERK1/2 and ERK 2 (E), tubulin (C), and actin (E). Western blots shown are representative of 3 separate experiments. In 12 h transwell chemotaxis assay, cells were pretreated with 50 μM LY 294002 (B) or 20 μM PD 98059 (D) for 45 minutes prior to the addition of APC (10 μg/mL). Cells migrated toward 10% FBS containing media for 12 h. Graphs represent the averages of 9 experiments for (B) and 8 experiments for (D). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to No Treatment; *aa* $p<0.01$ compared to APC treatment.

viability was measured using a MTT assay. MDA-MB-231 cell viability increased in the presence of EGF or EGF and mIgG compared to No Treatment (Figure 3.5B). This increase was abrogated by a blocking antibody to EGFR (20 μg/mL). In the transwell invasion and chemotaxis assay, APC increased invasion approximately 200% over No Treatment and chemotaxis approximately 150% (Figure 3.5C). Treatment with EGFR blocking antibody alone had no effect on cell migration (Figure 3.5C). In contrast, the EGFR blocking antibody substantially reduced APC-promoted invasion and chemotaxis of the MDA-MB-231 cells (Figure 3.5C). These results indicate that in addition to EPCR and PAR-1, a third receptor, EGFR, is involved in the APC-promoted chemotaxis and invasion of the MDA-MB-231 cancer cell line.

APC Promotes MDA-MB-231 Breast Cancer Invasion through MMPs. Having observed that MMPs are involved in APC mediated angiogenesis in our *ex vivo* aortic ring model, we investigated whether MMPs, specifically MMP-2 and/or MMP-9, are involved in the increased invasion of MDA-MB-231 cells in response to APC. It has previously been suggested that APC may directly activate MMPs or increase MMP expression [11-13] through NFκB activation [33, 34], which could occur downstream of PI3K and MAPK pathways. Our results suggest that despite NFκB activation, as evidence by degradation of the NFκB inhibitory protein IκB-α [35] (Figure 3.6A, inset), no statistically significant changes in MMP-2 or MMP-9 RNA occurred in response to APC. Furthermore, we did not see any evidence of increased protein levels of pro-MMP-2/9 or activated MMP-2/9 fragments as measured by immunoblot (data not shown).

Despite the lack of altered expression and activity of MMP-2/9 in response to APC, we reasoned they might still be involved in elevated invasion of MDA-MB-231 cells based on our results with the murine aortic ring assay. First, a broad-spectrum hydroxamate inhibitor of MMPs, GM 6001, was used to determine if MMPs have a role in APC promoted invasion of the

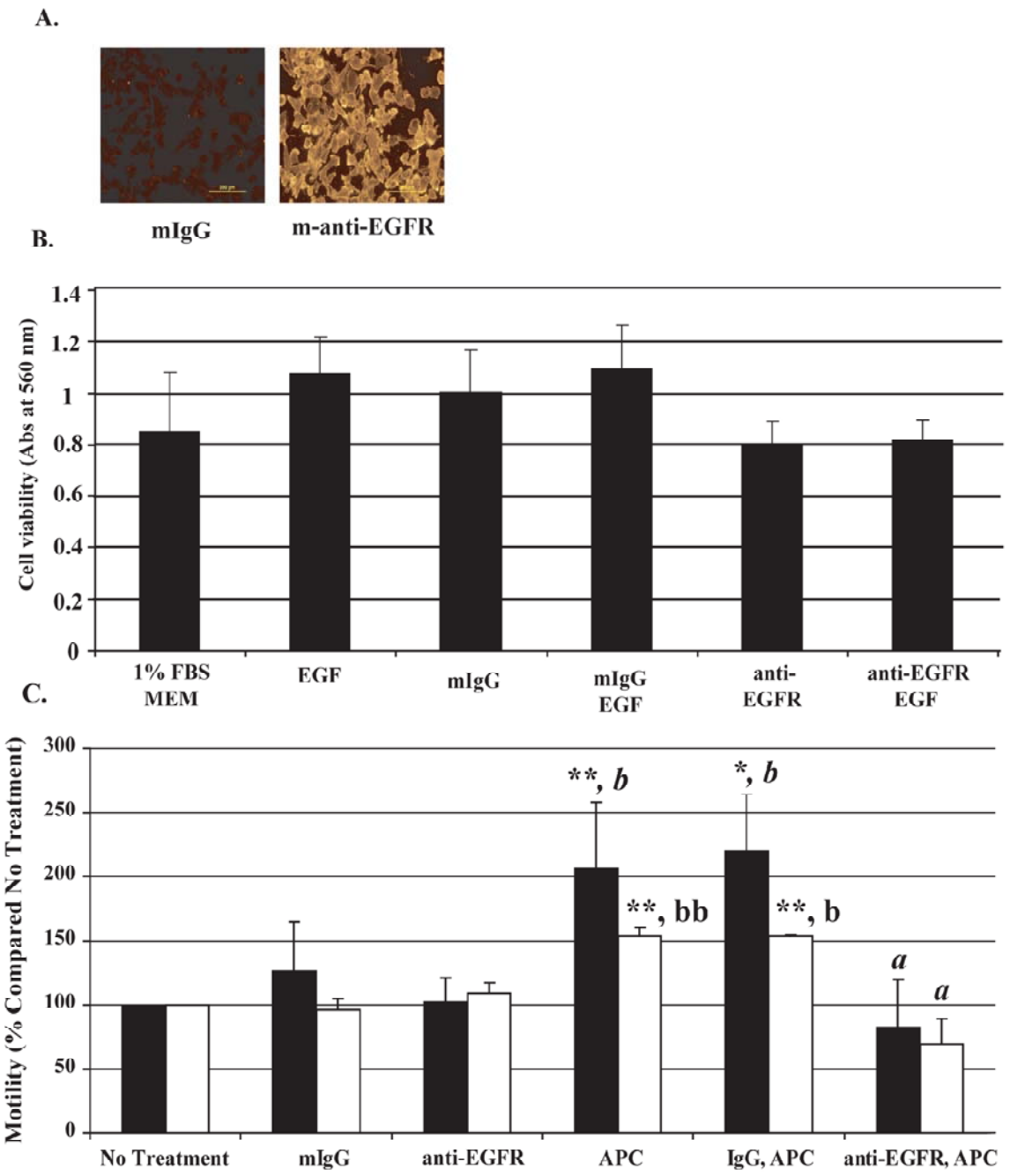

Figure 3.5. APC promoted invasion and chemotaxis of MDA-MB-231 cells is dependent upon activation of EGFR. (A) Immunofluorescence on the MDA-MB-231 cell monolayer for EGFR. (B) Cells were pretreated with EGFR antibody or control mouse serum IgG (20 μg/mL) prior to the addition of 1 nM EGF in SFM. Cells were allowed to grow for 48 h and then treated with MTT. Graphs represent the average absorbance for each condition for 4 separate experiments. (C) Cells were pretreated with 20 μg/mL anti-EGFR or control mouse serum IgG for 15 minutes prior to the addition of APC (10 μg/mL). Cells invaded for 24 h and chemotaxed for 12 h towards 10% FBS containing media. Graphs represent the average of 4 separate experiments for invasion and 3 separate experiments for chemotaxis assays; * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p<0.05$ compared to APC treatment; *b* $p<0.05$, *bb* $p<0.01$ compared to mIgG treatment.

MDA-MB-231 cells. As shown in Figure 3.6B, GM 6001 treatment reduced invasion compared to No Treatment in a concentration dependent manner as expected due to the reduction of active proteases that can degrade the ECM. GM 6001 and APC together yielded similar results. Because the GM 6001 MMP inhibitor is a broad-spectrum inhibitor, we also used an inhibitor with specificity for only MMP-2 and -9, SB-3CT, to determine if these MMPs were involved in APC-promoted cell invasion. Invasion was reduced in a concentration dependent manner upon SB-3CT treatment alone as expected (Figure 3.6C). With SB-3CT and APC treatment, we see a similar trend (Figure 3.6C). APC activity assays verified that neither GM 6001 nor SB-3CT had an effect on APC activity (data not shown). Combined with the data in Figure 3.6A, these results imply that MMP activity is necessary for APC induced invasion of MDA-MB-231 cells but that APC does not significantly increase MMP-2/9 activity or expression levels in these cells.

APC Does Not Increase MDA-MB-231 Breast Cancer Cell Invasion Through the Plasminogen Activation System. It was previously hypothesized that APC promotes invasion in ovarian and choriocarcinoma cells by altering the balance between uPA and PAI-1 to favor uPA proteolytic activity. If APC were to enhance uPA proteolytic activity, it is possible that the observed changes in PI3K, MAPK, and EGFR activity associated with APC treatment could be mostly due to uPA. We therefore examined this hypothesis with the MDA-MB-231 cell line which expresses both uPA and PAI-1. mRNA levels of uPA and PAI-1 were not altered in the presence of APC (data not shown). To determine if APC was altering the level of free uPA in the media, transwell invasion assays were performed with amiloride, a small molecule inhibitor of uPA, and a blocking antibody to uPA with APC. As shown in Figure 3.7A, APC or APC and DMSO increased invasion of the MDA-MB-231 cells greater than 150%. The amiloride treatment alone reduced invasion to approximately 70% of No Treatment. This result was

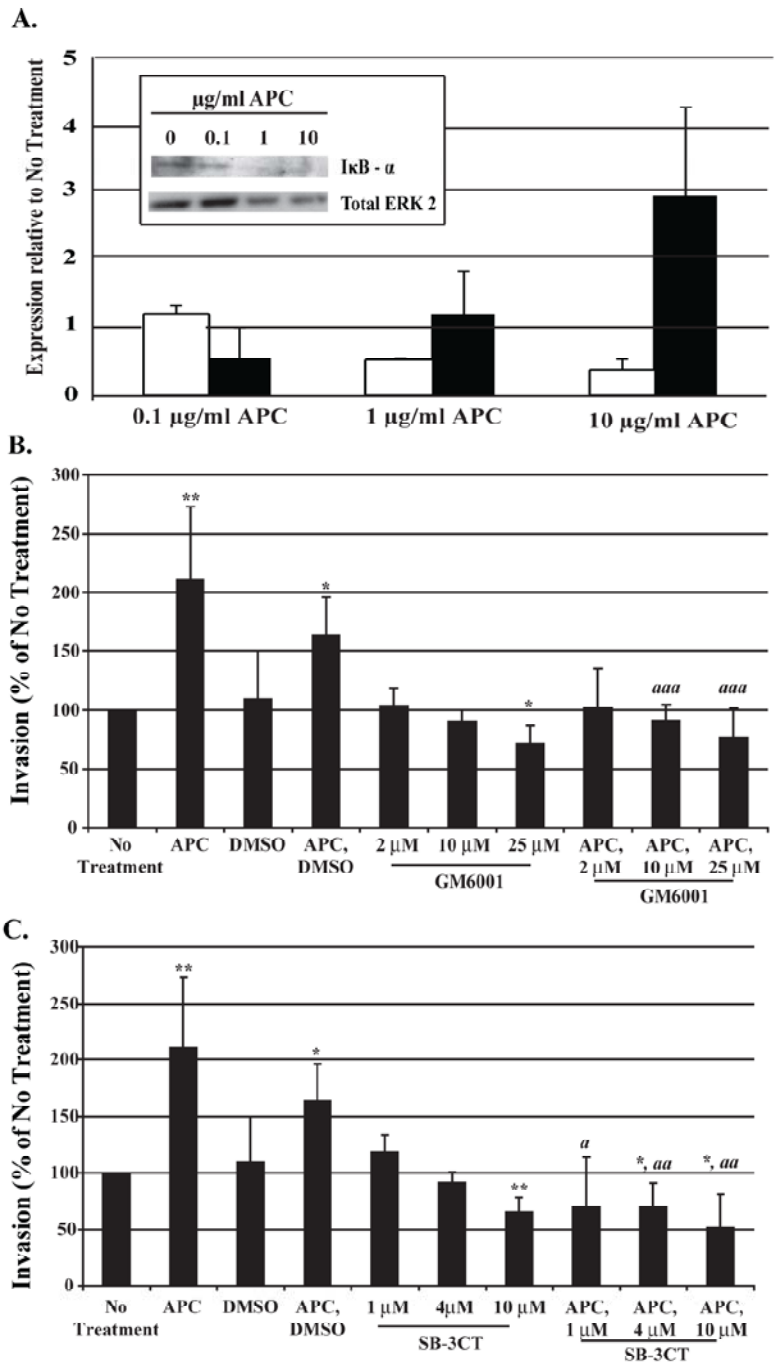

Figure 3.6: APC promoted invasion of MDA-MB-231 cells is dependent upon MMP-2 and MMP-9 degradation of the extracellular matrix. (A) Cells were treated with increasing concentrations of APC (0.1, 1, and 10 µg/ml) and assayed for MMP-2 and MMP-9 expression by quantitative PCR and NFκB activation by immunoblot of IκB-α (inset). For invasion assays, cells were pretreated with increasing concentrations of GM 6001 (0-25 µM; B) or SB-3CT (0-10 µM; C) for 15 minutes prior to the addition of 10 µM APC. Cells invaded towards 10% FBS containing media for 24 h. Graphs represent the averages of 8 separate experiments; * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p<0.05$, *aa* $p<0.01$, *aaa* $p<0.001$ compared to APC treatment.

expected since uPA was inhibited, reducing the activation of plasminogen and MMPs, which mediate cellular invasion. If uPA was a key component to the mechanism by which APC promoted invasion, then the treatment of APC and amiloride should have the same result as amiloride treatment alone. However, APC still increased invasion of the MDA-MB-231 cells even when uPA was inhibited, indicating that APC promotes cell invasion through a mechanism other than through uPA. APC activity assays verified that APC was not inhibited by amiloride (data not shown). To further confirm that uPA was not involved in the mechanism, a blocking antibody to the active site of uPA was used in the transwell invasion assay (Figure 3.7B). As was found with amiloride, even in the presence of the uPA blocking antibody, APC was able to increase cell invasion approximately 150% compared to No Treatment.

To determine if PAI-1 has a role in APC-promoted increase in invasion, a blocking antibody to PAI-1 was used along with APC in the transwell invasion assays (Figure 3.7C). In contrast to control goat IgG, the blocking antibody to PAI-1 increased invasion approximately the same amount compared to APC. With limited PAI-1 activity to inhibit uPA, the serine protease can more freely activate proteases that degrade the ECM, such as plasminogen and MMPs. Interestingly, the addition of APC with the blocking antibody to PAI-1 had an additive effect, increasing invasion further (to approximately 350% compared to No Treatment) over PAI-1 blocking antibody alone (Figure 3.1C). These results suggest that APC promotes invasion of the MDA-MB-231 cells by a mechanism distinct from altering the ratio between uPA and PAI-1 that favors uPA proteolytic activity.

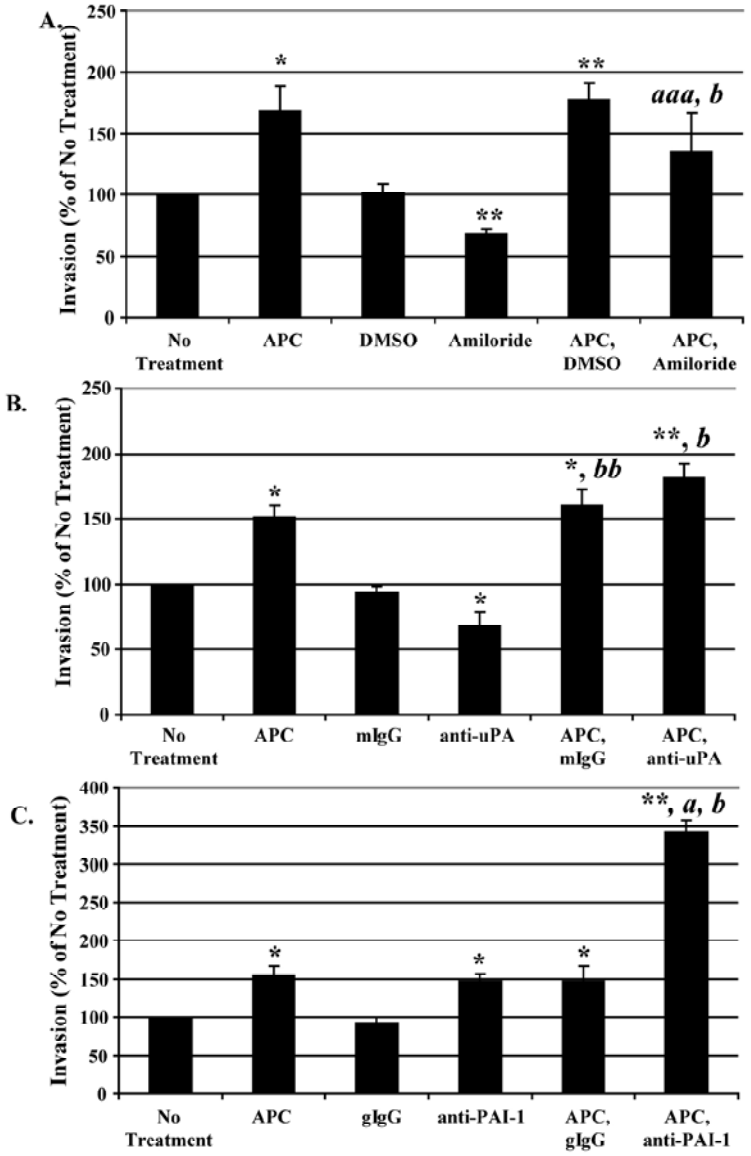

Figure 3.7: APC does not increase invasion of the MDA-MB-231 cells through the plasminogen activator system. Cells were pretreated with 0.2 mM amiloride (A), 20 μg/mL anti-uPA (B), 20 μg/mL anti-PAI-1 (C), and corresponding control antibodies for 1 h prior to the addition of 10 μg/mL APC. Cells invaded and migrated towards 10% FBS containing media for 24 h. Graphs represent the average of 3 separate experiments; * $p<0.05$, ** $p<0.01$ compared to No Treatment; *a* $p<0.05$, *aaa* $p<0.001$ compared to APC treatment; *b* $p<0.05$, *bb* $p<0.01$ compared to amiloride/anti-uPA/anti-PAI-1 treatment.

Discussion

In this study, we utilize *in vitro* and *ex vivo* models to show that APC can promote angiogenesis in both human and murine endothelial cells through MMP activation and EGFR transactivation – mechanisms that have been proposed for APC mediated motility in other cell types. Furthermore, based on previous findings and our own observations, we extended this examination to determine the mechanisms that govern APC promoted motility and invasion in MDA-MB-231 breast cancer cells. In both cell types, we found a common pathway whereby APC promotes angiogenesis and invasion, by activating MMPs and activating EGFR. Using blocking antibodies to EPCR, PAR-1, and EGFR with the breast cancer cells, we found that APC interacts with all three receptors to promote invasion. Our results imply that interaction of these three receptors with APC activates the PI3K and MAPK pathways and that activation of these pathways is necessary for APC to promote migration and invasion.

APC increases HUVEC invasion and migration at optimal concentrations of 0.1 μg/mL and 0.5 μg/mL, respectively. Furthermore, APC promotes tube like structures in HUVEC at 0.1 μg/ml. These two concentrations of active protease (0.1 μg/ml = 1.8 nM and 0.5 μg/ml = 8.8 nM) are significantly lower than zymogen PC in plasma (~4 μg/mL or ~71 nM), from which the active protease is derived, and are much less then what have been previously used to show that APC promotes HUVEC proliferation and tube formation [20]. However, these concentrations of APC are similar to those used previously to show that APC can regulate intracellular calcium fluxes [36]. Therefore, the effects of APC described here with HUVEC are likely relevant to physiological concentrations of protein C/APC. By contrast, while higher concentrations of APC were required to detect statistically significant changes in chemotaxis and invasion transwell

assays with the MDA-MB-231 cells, 0.1 μg/ml APC was sufficient to activate PI3K and MAPK signaling.

Besides activation of PI3K and MAPK signaling, we also observed activation of NFκB in the MDA-MB-231 cells in response to APC, a finding which contrasts observations in other cell types. Notably, APC has been shown to inhibit endotoxin induced NFκB nuclear translocation in monocytic cells [37] and APC down--regulates tPA induced NFκB activity in ischemic endothelial cells, thereby reducing MMP-9 activation [38]. The precise mechanism by which APC could instead activate NFκB in breast cancer cells remains unclear. However, cross-talk between PI3K, MAPK, and NFκB is possible, given the observation that breast cancer cells exhibit a unique positive correlation between PI3K/MAPK signaling and NFκB activation [39-41].

Using the murine *ex vivo* aortic ring assay, we show that APC increases sprout formation of vWF-positive endothelial cells and that this effect is dependent on MMP and EGFR activation. Notably, this phenomenon was only observed at a higher concentration of APC (10 μg/ml) than the optimal concentration observed for human cells in culture. In one study, the concentration of human APC that effectively reduced the volume of cerebral infarct and increased cerebral blood flow post-onset of stroke in mice was 2 mg/kg [42]. In contrast, similar studies done with murine APC showed that the same beneficial effects can be seen using 10 times less, 0.2 mg/kg [38, 43]. Therefore, it is likely that the requirement for higher concentrations in the murine aortic ring assay is a function of species differences, further suggesting that the effects of APC observed in this study are relevant to physiological concentrations of APC/PC.

Using pharmacological inhibitors in the transwell chemotaxis assays, along with western blot analysis, we show that APC activates the PI3K and MAPK pathways to increase motility of the MDA-MB-231 cells. Based on this study and our results of chemotaxis [23], we suggest that APC is focused at the cell surface through its binding to EPCR and that PAR-1 is subsequently cleaved by APC-bound to EPCR [38]. Furthermore, the results suggest that APC also transactivates EGFR, and that this activity is required to promote motility in the MDA-MB-231 cells.

There are many possible mechanisms whereby PAR-1 and EGFR could work in concert in response to APC to promote motility via activation of PI3K and MAPK signaling. First, APC could bind directly to EGFR, like recently shown for another serine protease, tissue plasminogen activator [44], and directly activate MAPK signaling through Ras [45]. Second, PAR-1 activation mediated by APC cleavage would lead to Gα or β/γ subunits activating c-Src. c-Src can directly activate PI3K and could also mediate phosphorylation of Tyr 845 on the cytoplasmic tail of EGFR [46], subsequently activating MAPK signaling via Ras. There is proof of principle for this mechanism. Via EPCR and PAR-1, APC has been shown to transactivate another receptor, sphingosine-1-phosphate$_1$ receptor, which affects vascular permeability of endothelial cells [47]. Third, PAR-1 could possibly activate the TNF-alpha converting enzyme (TACE; ADAM-17), a membrane-bound disintegrin metalloprotease that processes membrane-associated cytokines such as heparin bound-EGF (HB-EGF) or other EGFR-family member ligands to transactivate EGFR [48, 49]. HB-EGF would bind to the ligand-binding domain of EGFR, leading to the phosphorylation of the receptor and activation of Ras [50-52]. In any mechanism in which Ras is activated, there is a potential for PI3K activation as Ras has been shown to directly activate PI3K [53]. Our results do not favor the direct activation of EGFR by APC, but

suggest that APC-EPCR interactions through PAR-1 promote either the transphosphorylation of EGFR (since the EGFR blocking antibody reduced motility in the presence of APC) or by ADAM-17-directed ligand transactivation (since the MMP inhibitor GM 6001 is also known to inhibit ADAM-17).

Within the context of the MDA-MB-231 breast cancer cells, our results suggest a shift away from other mechanistic paradigms that suggest the primary role of APC in motility involves the regulation of pericellular proteolysis. There is prior evidence that APC directly activates MMPs, specifically MMP-2. Through zymography, APC was also shown to activate MMP-2 from the intermediate to fully active form [16, 18, 54], independent of MT1-MMP [16, 54]. Additionally, it has also been shown that solution phase APC [16, 54] and APC generated on the cell surface in the presence of thrombin bound to TM can activate MMP-2 [18]. Furthermore, it has been suggested that APC can promote increased expression of MMP-2 [17, 19]. In our study we found that MMP activity, specifically MMP-2 and MMP-9 activity, is required for APC to promote invasion. However, we did not observe significant changes in either MMP-2 or MMP-9 RNA expression. We also did not observe differences in protein levels of pro-MMP-2/9 or any difference in the amount of active MMP2/9 in culture media in response to APC. These results suggest that while some basal level of MMP-2/9 activity is required to initiate pericellular proteolysis of the extracellular matrix in response to APC, treatment with APC is not significantly increasing MMP expression or activity in the MDA-MB-231 cells. It is likely that high levels of uPA as well as signaling cascades that can promote MMP expression, such as MAPK signaling, lead to high basal activity and expression of MMP-2/9 that is not significantly altered by APC treatment.

In a similar regard, our results with the MDA-MB-231 cells also differ from previous studies which suggest that APC promotes motility through inhibition of PAI-1 with subsequent activation of uPA. Kobayashi, et al. [10] found that exogenously added APC increased invasion of cancer cells *in vitro* by forming a stable complex with the serpin, PAI-1 and thereby APC promotes invasion by increasing uPA activity [10]. However, in our study, blocking uPA with either the chemical inhibitor amiloride or a blocking antibody to its active site did not alter the ability of APC to promote invasion of the MDA-MB-231 breast cancer cells. Moreover, in the presence of a PAI-1 blocking antibody and APC, there was an additive effect to promote invasion. The observation that APC does not promote motility through the plasminogen activator system, taken together with our findings about the role of MMP's in breast cancer cell motility, suggests that APC promotes motility of the MDA-MB-231 cells largely as a consequence of its ability to activate EGFR, MAPK, and PI3K associated signaling cascades.

In summary, our results show that APC promotes endothelial motility via activation of EGFR and MMP's, both *in vitro* and *ex vivo*. With regard to our unique findings with the MDA-MB-231 cells, our results show an interesting interdependent nature of the pathways that are activated by APC to increase motility. Blocking of any one of the three involved receptors, (EPCR, PAR-1 or EGFR), or inhibition of MMP-2 and/or MMP-9 results in a loss of the increase in motility promoted by APC. Overall, the effect of APC is coordinated with both intracellular activation of signaling pathways, which promote motility, and with some level of pericellular activation of MMPs, which primes the cells to respond to chemoattractants at an increased rate.

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Chapter IV

Plasminogen Activator Inhibitor-1 is an Aggregate Response Factor with Pleiotropic Effects on Cell Signaling in Vascular Disease and the Tumor Microenvironment

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Abstract

In hemostasis, the serine protease inhibitor (serpin) plasminogen activator inhibitor-1 (PAI-1) functions to stabilize clots via inhibition of tissue plasminogen activator (tPA) with subsequent inhibition of fibrinolysis. In tissues, PAI-1 functions to inhibit extracellular matrix degradation via inhibition of urokinase plasminogen activator (uPA). Elevated levels of PAI-1 in the vasculature and in tissues have long been known to be associated with thrombosis and fibrosis, respectively. However, there is emerging evidence that PAI-1 may participate in the pathophysiology of a number of diseases such as atherosclerosis, restenosis, and cancer. In many of these disease states, the canonical view of PAI-1 as an inhibitor of tPA and uPA cannot fully account for a mechanism whereby PAI-1 contributes to the disease. In these cases, one must consider recent data, which indicates PAI-1 can directly promote pro-proliferative and anti-apoptotic signaling in a variety of cell types. Given the wide variety of inflammatory, hormonal, and metabolic signals that increase PAI-1 expression, it is important to consider mechanisms by which PAI-1 can directly participate in disease etiology.

Introduction

Traditional paradigms for PAI-1 in fibrinolysis and tissue remodeling

Within the intravascular space, the primary role for the serpin (serine protease inhibitor) PAI-1 is to regulate fibrinolysis to stabilize hemostatic plug formation. When bound to fibrin in a clot, the serine protease tissue plasminogen activator (tPA) activates plasminogen to its active form of plasmin, which subsequently degrades fibrin [1]. During thrombus formation, tPA is inhibited by PAI-1 released from platelets [2], thereby limiting further plasminogen activation and fibrinolysis.

The primary role of PAI-1 in the extravascular space is to regulate matrix remodeling via inhibition of the urokinase plasminogen activator (uPA) [3]. uPA bound to cells expressing its cognate receptor uPAR, can catalyze the pericellular conversion of plasminogen to plasmin, which can subsequently cleave and/or activate numerous proteins such as gelatinase, fibronectin, laminin, and latent forms of collagenases including MMP-1 to lead to matrix degradation. As with tPA, PAI-1 forms a 1:1 complex with uPA and renders the protease inactive, thereby inhibiting pericellular proteolysis.

PAI-1 has also been implicated in inhibiting adhesion of cells to extracellular matrix proteins, although the precise mechanism remains debated. uPAR has been shown to associate with multiple different integrin subunits and it has been suggested that uPAR can act as an integrin ligand to promote both cell adhesion to various matrix proteins [4], as well as cell to cell adhesion [5]. Given these findings, it has been suggested that PAI-1 may promote de-adhesion from various substrata via destruction of integrins [6]. Alternatively, is has been suggested that PAI-1 can promote de-adhesion specifically for the extracellular matrix protein vitronectin (VN) [7]. PAI-1 and uPAR/uPA complexes compete for binding

to VN [7], and binding of PAI-1 to uPA dissociates uPAR from vitronectin. Endocytosis of the uPAR/uPA/PAI-1 ternary complex by the low-density lipoprotein-like receptor 1 (LRP-1) then promotes de-adhesion of cells from VN [8].

Regulation of PAI-1 expression in the intravascular and extravascular space

During the initiation of thrombus formation, release of PAI-1 by platelets represents the most likely primary source of PAI-1 [2]. However, multiple cell types are capable of producing PAI-1 in response to various inflammatory cytokines. The multiplicity of potential sources of PAI-1 as a response factor has implications for PAI-1 function in both physiological and pathophysiological conditions.

As a recognized acute phase reactant, PAI-1 levels in plasma increase quickly in response to vessel injury and a heightened inflammatory state [9]. Like C-reactive protein (CRP) and fibrinogen, PAI-1 levels in plasma have been shown to increase in response both to acute trauma such as local tissue injury [10] and to chronic inflammatory states such as cardiovascular disease [11] and insulin resistance [12]. In mouse models, this increase has been attributed to increased synthesis of PAI-1 by the liver in response to inflammatory cytokines IL-1β [10], IL-6 [13], and tumor necrosis factor-α (TNFα) [14]. Under physiological conditions, acute increases in the plasma concentration of PAI-1 in response to inflammatory cytokines could be viewed as a mechanism to stabilize thrombus formation by inhibiting tPA-mediated plasminogen activation. However, under pathological conditions such as atherosclerosis, sustained elevated levels of PAI-1 could promote thrombosis.

In contrast to fast up-regulation of PAI-1 in plasma as an acute phase reactant, there are also mechanisms by which PAI-1 levels in the intravascular space may be up-regulated in

a more sustained fashion via endothelial cell production. Similar to hepatocytes, cultured endothelial cells have been shown to increase PAI-1 production in response to the inflammatory cytokines IL-1 [15] and TNF-α [16]. PAI-1 synthesis by endothelial cells has also been shown to increase as a consequence of hypoxia [17], the generation of reactive oxygen species [18], and shear stress [19]. Alternatively, increased PAI-1 production by endothelial cells has also been associated with senescence, a process that increases with age [20].

Extravascularly, regulation of PAI-1 expression involves multiple cell types. In fibroblasts, PAI-1 synthesis is increased in response to TGF-β [21] and IL-6 [22]. Perhaps the most important source of PAI-1 in tissues is adipocytes. Mature adipocytes express relatively high basal levels of PAI-I in culture [23]. Despite high basal expression, adipocytes can increase PAI-1 synthesis in response to many cytokines and hormones such as TNF-α, TGF-β, and insulin [24]. Finally, macrophages represent another source of PAI-1 in the extravascular space. Activation of monocytes with endotoxin increases PAI-1 expression and histological studies indicate that tissue macrophages in artheromatous plaques express PAI-1 [25].

Given the diversity of cellular sources and multitude of inflammatory signals which promote PAI-1 expression, it is not surprising that elevated PAI-1 levels in serum and tissue have been observed in a variety of pathological conditions. One might suggest elevated PAI-1 levels could be merely a marker of inflammation. However, multiple studies have shown that PAI-1 participates directly in the pathophysiology of a number of diseases. In some cases, the traditional paradigms for the function of PAI-1 can fully explain its role in pathology. Alternatively, in other disease states, the traditional paradigms for the function of

PAI-1 are not sufficient to understand its participatory role. Thus, new data are emerging that strongly suggests PAI-1 has novel functions far beyond its ability to inhibit tPA and uPA.

Pathologic consequences of PAI-1 expression explained by traditional paradigms

PAI-1 in thrombosis- As an inhibitor of plasminogen activation and fibrin degradation, it is logical that elevated PAI-1 levels in serum would lead to thrombosis. With regard for thrombosis in the coronary arteries, elevated PAI-1 levels have been documented in the serum of survivors of a myocardial infarction and patients that have recurrent myocardial infarctions [26]. In an experimental model, transgenic mice that express a stable form of human PAI-1 develop spontaneous coronary thrombi [27]. Elevated levels of PAI-1, especially in the elderly, are thought to be associated with both venous and arterial thrombosis [28].

PAI-1 in fibrosis- In tissues, increased expression of PAI-1 has been associated with multiple forms of fibrosis including glomerulosclerosis [29], liver fibrosis [30], and pulmonary fibrosis [31]. While there are competing schools of thought on the role of PAI-1 in promoting fibrosis, all utilize the traditional paradigm for PAI-1 function. First, it is thought that elevated PAI-1 expression decreases tPA/uPA activity leading to increased fibrin deposition at the site of a vessel injury. Due to the increased fibrin deposition, more cells infiltrate the wound, leading to increased collagen deposition. In an alternative model, it has been suggested elevated PAI-1 promotes de-adhesion, allowing for more cells to infiltrate. Finally, it has also been suggested that the primary consequence of elevated PAI-1

is actually decreased collagenase activity downstream of reduced uPA and MMP activation. In this model PAI-1 directly promotes fibrosis by inhibiting collagen degradation [32].

In contrast to observations linking PAI-1 to thrombosis and fibrotic disease, the role of PAI-1 in other pathological conditions are not explained by traditional paradigms, which focus solely on the protease inhibitor activity of PAI-1. More recent studies elucidating the ability of PAI-1 to alter cell signaling is providing insight to explain how PAI-1 can contribute to other disease states.

Pathologic consequences of PAI-1 expression <u>not</u> explained by traditional paradigms

PAI-1 in vascular disease- PAI-1 has a well-documented association with the development of vascular disease. Multiple studies have demonstrated the presence of excess PAI-1 in atherosclerotic plaques [25, 33, and 34]. Furthermore, PAI-1 deposition in the vascular wall [35], and atherosclerotic plaques [36], is elevated in patients with type II diabetes. This is not surprising given that elevated PAI-1 expression has been directly linked to hyperinsulinemia as well as glucose/lipid imbalance [37].

It has been suggested that the presence of PAI-1 in atherosclerotic plaques may directly contribute to atherogenesis primarily via inhibition of MMP activation with subsequent decreased activation of TGFβ [38], which promotes smooth muscle cell proliferation, and decreased processing of cholesterol aggregates [26]. However, there are other mechanisms by which PAI-1 may promote the development of atherosclerosis that reach beyond the traditional paradigms of tPA/uPA inhibition. Of note, PAI-1 has shown to directly promote migration, inhibit apoptosis [39], and promote proliferation [40] of smooth muscle cells. These studies elucidate that PAI-1 has unique capabilities to promote cell

signaling. With regard to apoptosis and proliferation, these studies show purified PAI-1 inhibits caspase-3 activity *in vitro* [39], and promotes cell proliferation via activation of the NFκB and ERK signaling [40]. Given the importance of smooth muscle cell proliferation in the development of atherosclerotic lesions, it is important to consider the potential role of PAI-1 directly mediating intracellular signaling events in vascular disease.

Another interesting potential mechanism of PAI-1 mediated signaling in vascular disease involves macrophages recruitment. In *in vitro* studies, PAI-1 in combination with tPA and LRP-1 (the co-receptor for clearance of tPA-PAI-1 and uPA-PAI-1 complexes), has been shown coordinate Mac-1 dependent macrophage migration [41]. While the authors of these studies suggest PAI-1 mediated migration of macrophages is a function of the de-adhesive functions of PAI-1, it is likely to involve more complicated signaling events in light of data which shows the LRP-1 co-receptor acts as a motogenic receptor for PAI-1, and can activate Jak/Stat signaling when it binds PAI-1 [42].

PAI-1 in the tumor microenvironment

The tumor microenvironment represents a heterogeneous mixture of cell types, many of which can produce and respond to PAI-1, including the tumor cells themselves. Considering the well documented role of the immune system and inflammation in cancer and the tight linkage between inflammation and elevated PAI-1, one might hypothesize that PAI-1 could play a direct role in the pathophysiology of cancer. This hypothesis should garner close investigation given that elevated PAI-1 levels in the tumor microenvironment correlates to poor prognosis in multiple cancers including breast [43], ovarian [44], pulmonary adenocarcinoma [45], and neuroblastoma [46]. The commonly observed elevation of PAI-1

in cancer presents a difficult paradox. Given the well-documented association of matrix degradation with tumor viability and metastasis, one would expect that PAI-1, as an inhibitor of uPA, would represent a positive prognostic indicator. However, insights into the novel functions of PAI-1 as a cell signaling mediator may help explain why PAI-1 is often a poor prognostic indicator.

Most directly, PAI-1 produced by adipocytes, fibroblasts, or macrophages in response to various cytokines in the tumor microenvironment can promote angiogenesis, which is crucial to tumor viability and dissemination. While not without controversy, PAI-1 is generally accepted to promote angiogenesis in the tumor microenvironment. Utilizing PAI-1$^{-/-}$ mice, it has been shown PAI-1 promotes angiogenesis in the tumor microenvironment at low concentrations but inhibits angiogenesis at extremely high concentrations [47]. The precise mechanism by which PAI-1 promotes angiogenesis has remained largely elusive, although it has been suggested that the ability of PAI-1 to promote angiogenesis is dependent on it protease inhibitor activity, not vitronectin binding [48]. This finding would suggest PAI-1 does not promote angiogenesis by promoting de-adhesion of endothelial cells from matrix proteins. The most complete study analyzing angiogenesis suggests that PAI-1 inhibits pro-apoptotic cell signaling by inhibiting plasmin mediated cleavage of Fas-ligand on the surface of endothelial cells [49]. This represents a novel mechanism for how PAI-1 prevents apoptosis of endothelial cells and therefore promotes angiogenesis.

Another mechanism whereby PAI-1 could function to promote cancer dissemination comes from a novel finding with senescence in fibroblasts. PAI-1 has long been known as a marker of senescence in multiple cell types [50, 51]. However, it has been shown that PAI-1 is not only a marker of senescence in fibroblasts, but that it is a critical downstream target of

p53 in the induction of senescence, through a PI3K-dependent pathway [52]. If PAI-1 can participate directly in the induction of replicative senescence, it is possible that an excess of PAI-1 in the tumor microenvironment could induce the senescence of fibroblasts, leading to decreased deposition of matrix.

Outside of its influence on endothelial cells and fibroblasts in the tumor microenvironment, emerging data suggests PAI-1 has direct influence over pro-proliferative and anti-apoptotic signaling in tumor cells themselves. While mouse models designed to examine the effect of PAI-1 on tumor development have yielded often-conflicting data [53], *in vitro* examination of various cancer cell lines indicates that PAI-1 has a positive influence on cancer cell growth and survival. While limited, this data may provide the most direct link to explain why elevated PAI-1 levels are associated with poor prognosis in cancer.

One such study demonstrated that both the PC-3 prostate cancer cell line and the HL-60 promyelocytic cell line demonstrated decreased apoptosis in the presence of PAI-1 when treated with apoptosis inducing agents camptothecin or etoposide [54]. Similarly, our laboratory has found that stable expression of wild type PAI-1, but not an inactive mutant of PAI-1, enhances the recovery of MDA-MB-435 cancer cells after exposure to apoptosis inducing agent paclitaxel [55], and enhances motility and adhesion via alteration of the integrin profile at the cell surface [56]. Fibrosarcoma cell lines derived from PAI-$1^{-/-}$ mice were significantly more sensitive to apoptotic stimulus etoposide than counterpart fibrosarcoma cell lines from wild type mice [57].

More recent data reveals how PAI-1 may influence cancer cell viability via direct alteration of cell signaling. An expanded analysis of the murine fibrosarcoma cells discussed above revealed that PAI-1 deficient fibrosarcomas have reduced Akt signaling and re-

introduction of PAI-1 expression in deficient cells results in an increase in Akt signaling [58]. Our laboratory has found that stable knockdown of PAI-1 expression in MDA-MB-231 breast cancer cells results in decreased Akt activation (Gramling, M.W. and F.C. Church, unpublished data). Besides regulation of anti-apoptotic Akt signaling, PAI-1 has also been shown to activate pro-proliferative MAPK signaling. In MCF-7 breast cancer cells, PAI-1 promotes sustained phosphorylation of ERK1/2 via a mechanism which is dependent on its interaction with uPA as well as the activity of uPAR and the LRP-1 co-receptor (59). This study further showed that the presence of PAI-1 allowed uPA to act as a mitogen for the breast cancer cells [59].

Conclusion

Given that PAI-1 expression is elevated both the intra- and extra-vascular space by such a wide variety of inflammatory, hormonal, and metabolic signals, it has potential to influence many physiological and pathophysiological conditions. The pleiotropic effects of PAI-1 are diagrammed in Figure 4.1. From thrombosis and fibrosis to atherosclerosis and cancer, PAI-1 directly contributes to the etiology of disease via mechanisms explained by traditional paradigms of its function and by new mechanisms explained by its emerging role in altering cell signaling.

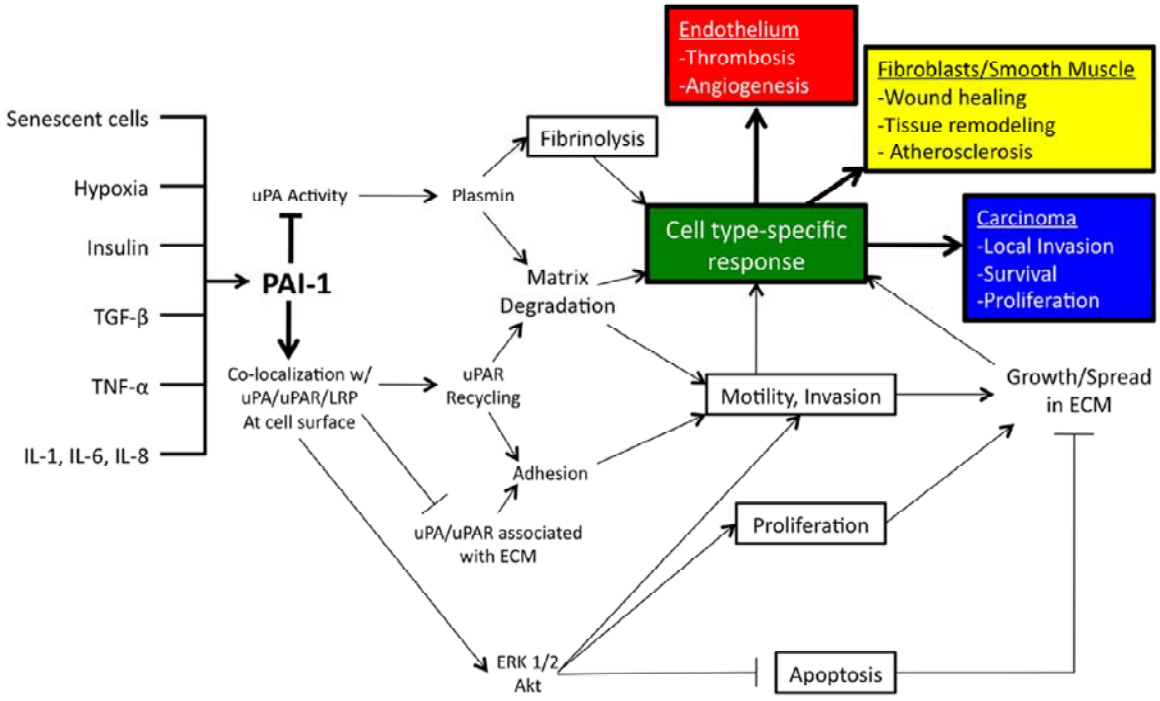

Figure 4.1. PAI-1 has been implicated in modulating a variety of physiological and pathophysiological processes. PAI-1 expression in the intra- and extra-vascular space is increased in response to many inflammatory, hormonal, and metabolic signals. By modulating protease activity, adhesion, and altering signaling in multiple cell types, PAI-1 can contribute to many normal and disease processes.

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Chapter V

Urokinase-type Plasminogen Activator and Plasminogen Activator Inhibitor 1 Modulate Proliferation and Sensitivity to Apoptosis in Breast Cancer Cells

Manuscript submitted for publication

Abstract

Both urokinase plasminogen activator (uPA) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1), are clinically recognized markers of poor prognosis in breast cancer. In this study we examined if uPA and PAI-1 can directly contribute to the cellular pathophysiology of breast cancer cells utilizing MDA-MB-231 and MDA-MB-436 cells as a model system. uPA and PAI-1 activity were inhibited, and shRNA constructs targeted against uPA and PAI-1 RNA were used to establish stable knockdown in MDA-MB-231 cells. Catalytic inhibition of both uPA and PAI-1 activity inhibited MAPK and PI3K activities and reduced growth in a three dimensional extracellular matrix (ECM). uPA and PAI-1 inhibitor treatments of both MDA-MB-231 and MDA-MB-436 breast cancer cells inhibited cell growth in low serum conditions. As expected, MDA-MB-231 cells with stable knockdown of uPA exhibited reduced MAPK activity and reduced growth in low serum conditions relative to cells carrying a non-targeted lentivirus (pLKO), but were not sensitized to chemotherapeutic agents. MDA-MB-231 cells with PAI-1 knockdown exhibited reduced PI3K activity and MAPK activity and displayed a similar growth defect in low serum and in a three dimensional ECM. To more closely assess the cause of the unique growth defects observed for PAI-1 knockdown cells, their basal proliferative capacity *versus* sensitivity to apoptosis was examined. PAI-1 knockdown did not lead to reduced staining for proliferation marker Ki67 but did result in increased staining for the apoptosis marker active Caspase-3. PAI-1 knockdown led to sensitization of cells to chemotherapeutic agents paclitaxel, doxorubicin, and cisplatin. Furthermore, PAI-1 inhibitor treatment of MDA-MB-436 cells caused similar sensitization to all three chemotherapeutic agents. Collectively, these results suggest that PAI-1 may protect cancer cells from apoptosis thereby directly participating in the cellular pathophysiology of breast cancer.

Introduction

The plasminogen activator (PA) system is well documented as a key player in the processes of matrix remodeling and cell motility [1]. At the cell surface, the serine protease urokinase plasminogen activator (uPA) is bound to its cognate receptor uPAR. This interaction promotes the ability of uPA to activate pericellular plasminogen to its active form, plasmin [2] which can degrade multiple matrix proteins including fibronectin and laminin as well as activate multiple matrix metalloproteinases (MMPs) [3]. In addition, uPA and uPAR are thought to directly promote cell motility with the interaction of uPAR with integrins [4], uPA mediated activation of multiple components of focal adhesion components [5], and specific binding of uPAR to the extracellular matrix (ECM) protein vitronectin [6]. The primary physiological inhibitor of uPA is PAI-1 [7,8]. When the reactive center loop of PAI-1 binds to the active site of uPA, a covalent 1:1 stoichiometric complex is formed. The trimeric complex of uPAR/uPA/PAI-1 is subsequently bound by the low density lipoprotein like receptor 1 (LRP-1), which catalyzes endocytosis of the entire complex [9,10]. Notably, PAI-1 also binds vitronectin [11,12] and competes against uPAR mediated adhesion to vitronectin [13].

Given the contributions of uPA/uPAR to proteolysis and cell motility it is logical that elevated uPA/uPAR expression would be associated with increased metastasis and therefore poor prognosis. In fact, this has been demonstrated in esophageal, gastric, colorectal, small cell lung, osteosarcoma, ovarian, prostate, and breast cancer among others [14]. This association is complemented by studies demonstrating that uPA/uPAR can stimulate both

proliferative and anti-apoptotic signaling [15,16]. However, a paradox emerges regarding the PA system's association with cancer because PAI-1 elevation is also associated with poor prognosis in a number of cancers including ovarian [17], pulmonary adenocarcinoma [18], neuroblastoma [19], and breast [20]. Effectively, if the physiological role of PAI-1 is to inhibit uPA protease activity, it is unclear how elevated PAI-1 could be associated with poor prognosis in the context of metastatic potential and proliferative/anti-apoptotic signaling. This "PAI-1 Paradox" is especially important in breast cancer, as elevation of both uPA and PAI-1 has been demonstrated to be a strong predictor of poor prognosis in breast cancer at the highest levels of significance using a level of evidence 1 (LOE-1) study [21].

In seeking to understand the PAI-1 paradox in tumor biology, previous studies have focused on its role in angiogenesis and cell motility. PAI-1 appears to promote angiogenesis in a dose dependent manner and prevent apoptosis in endothelial cells [22-25]. Others have sought to explain the PAI-1 paradox by showing that PAI-1 may directly promote motility of cancer cells by balancing the progressive steps of adhesion and de-adhesion required for cell motility [26].

Given the strong correlation between expression of uPA and PAI-1 and prognosis in breast cancer, in this study we aim to elucidate the direct effects of uPA and PAI-1 on cancer cell proliferation and apoptosis using primarily MDA-MB-231 breast cancer cells. These cells are an optimal cell system to examine the role of the PA system in breast cancer cell biology because they are unique among common breast cancer cell lines in their expression of high levels of both uPA and PAI-1 [27]. In some experiments we utilized the MDA-MB-436 breast cancer cell line. While this cell line is less well characterized than MDA-MB-231 cells, they are reported to make comparable levels of uPA and PAI-1 [27]. The expression of

both uPA and PAI-1 by the cells allows us to examine the effect of parallel enzymatic uPA and PAI-1 inhibition as well as parallel stable knockdown of uPA and PAI-1, and to assess the downstream consequences on both proliferation and apoptosis in multiple experimental contexts.

Materials and Methods

Cell Culture - MDA-MB-231 and MDA-MB-436 cells were obtained from ATCC. MDA-MB-231 cells were maintained in Minimum Essential Media (MEM; Gibco) with 10% FBS (Sigma), 1% sodium pyruvate (Gibco), and 1% antibiotic/antimycotic (Gibco). MDA-MB-436 cells were maintained in MEM with high glucose (Gibco) supplemented with 10% FBS (Sigma), 10 μg/ml insulin (Gibco), 2 mM glutathione (MB Biomedicals), 1% sodium pyruvate (Gibco), and 1% antibiotic/antimycotic. All cells were cultured in an incubator at 37 °C, 5% CO_2. For MDA-MB-231 cells carrying lentiviral shRNA, media was supplemented with 7.5 μg/ml puromycin (Sigma).

Cell Invasion Assays - Invasion assays with MDA-MB-231 cells were performed using BD BioCoat™ Matrigel™ Invasion Chambers (BD Bioscience), with an 8-μm diameter pore size membrane. Matrigel™ coated membranes, were hydrated in serum-free medium containing 0.1% bovine serum albumin (BSA) for 2 h at 37°C before the experiment. As a chemoattractant, complete media with 10% FBS was added to the lower well of the plate. Cells were pre-treated in serum free media for 1 hr with 20 μg/ml goat inhibitory PAI-1 antibody (395G, American Diagnostica), mouse inhibitory uPA antibody (394, American Diagnostica), serum mouse or goat IgG (15256, Sigma), as well as the small molecule uPA

inhibitor amiloride (A7410, Sigma) at 0.2mM, seeded at 5×10^4 cells in the culture insert with treatments, and incubated for 24 hr at 37°C, 5% CO_2. The number of cells that migrated through the membrane was counted via Hoechst staining as described previously [28].

Growth Assays and Chemotherapeutic Sensitivity Assays - For 7 day growth assays, 1×10^4 cells were plated in triplicate in 96 well plates in complete media. After 24 hr, cells were washed with PBS and treated with 1% serum medium containing control DMSO or amiloride and control goat IgG (Sigma) or goat PAI-1 inhibitory antibody (395G American Diagnostica). Viability was assessed 24 hr post plating and at days 1, 3, 5, and 7 post treatment by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [29]. Medium with or without inhibitors was replaced every 48 hr. For treatments with chemotherapeutic agents, 2.5×10^4 cells were plated in triplicate in 96 well plates. Cells were serum starved for 24 hr, treated with complete medium containing 100 μM paclitaxel, 10 μM doxorubicin, or 10 μM cisplatin for 48 hr, and then assayed by MTT. For MDA-MB-436 cells, cells were treated with 1 μM paclitaxel, doxorubicin, or cisplatin in the presence of either 20 μg/ml goat IgG or goat PAI-1 inhibitory antibody.

Three Dimensional Culture - Cells were grown in growth factor-reduced basement membrane extract (Cultrex) with protocols adapted from the "3D on top" method of Bissel and colleagues [30]. Briefly, 24 well plates were coated with 120 μl of basement membrane extract. Sub-confluent cells were trypsinized and re-suspended in complete medium supplemented with 10% basement membrane extract with or without uPA and PAI-1 inhibitory treatments and 3×10^4 cells were seeded onto the wells. Cells were maintained for 6 days with re-supplementation of medium containing 10% basement membrane extract plus

or minus treatment every 48 hours. Photomicrographs were obtained on day 6 with and Olympus CK40 inverted microscope.

Immunoblot Analysis – After 24 hours of serum deprivation, whole cell lysates, or conditioned medium concentrated by centrifugal filtering devices (Millipore) with a molecular weight cutoff of 30 kDa, were separated on 7.5% SDS-PAGE gels and transferred onto PVDF membranes (Millipore). Whole cell lysates were probed for P-ERK1/2 (E-4, Santa Cruz Biotechnology), total ERK2 (C-14, Santa Cruz Biotechnology), P-Akt (9271, Cell Signaling), or total Akt (9272, Cell Signaling). Concentrated conditioned medium was probed for uPA (American Diagnostica) or PAI-1 (Molecular Innovations). Densitometry of immunoblots was performed using ImageJ software from NIH and results are expressed as fraction of phosphorylated signal relative to total ERK2 or Akt. In experiments assessing the effects of purified uPA, PAI-1, or uPA-PAI-1 complex, 50% confluent cells were subjected to a mild acid wash to remove cell surface uPA, and allowed to recover in complete medium for 18 hr [31]. Cells were then washed 3X in PBS and then treated with 10 nM uPA (Calbiochem), 20 nM stable recombinant PAI-1 (purified as previously described [32]), or uPA-PAI-1 complex formed by 30 min incubation of a 1:2 molar ratio of uPA and recombinant stable PAI-1.

Indirect cell surface uPA activity assay - 1 x 10^4 cells were plated in 96 well plates and plasminogen activation was assessed as previously described [33, 34]. Plasminogen (0.06 μg/uL) (Chromogenix, Milan, Italy) in 100 mM Tris, 0.5% Triton X-100 (pH 8.8) was added to cells and incubated at room temperature for 30 min. Following incubation, 5 μl of supernatant was removed and added to another 96-well plate containing 74.8 μl buffer (100 mM Tris, pH 8.8 with 0.5% Triton X-100) and 5 μl amiloride (6 mM stock solution) to

inhibit residual uPA activity. Plasmin chromogenic substrate (S-2251, Chromogenix, Milan, Italy) was then added to the well (100 μL total volume) and hydrolyzed by plasmin generated by plasminogen cleaved by uPA on the cell surface. The relative rate of plasmin activity was determined from the rate of color development at 405 nm.

Lentiviral small hairpin RNA - pLKO.1, a nontargeted small hairpin control vector (Sigma) or shRNA vectors directed at uPA NM_002658 (TRCN0000051088, TRCN0000025089, TRCN0000025090, TRCN0000025091, TRCN0000025092) or PAI-1 NM_000602 (TRCN0000052268, TRCN0000052269, TRCN0000052270, TRCN0000052271, TRCN000002272) were converted into lentiviral particles by the UNC-CH Vector Core Facility according to the protocol of Kafri and collegues [35]. For infection, cells were incubated with lentiviral particles supplemented with Polybrene and stably infected populations were selected and maintained in puromycin. Knockdown efficiency for all sequences was assessed by quantitative PCR. Populations infected with TRCN0000025901 (designated uPAshRNA#4) as well as TRCN000052271 and TRCN000002272 (designated PAI-1shRNA#4 and PAI-1shRNA#5, respectively) were expanded for analysis.

Quantitative PCR - Total RNA was isolated from serum starved cells via Trizol (Invitrogen), RNA was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using SYBR Green Dye (Applied Biosystems) and the following protocol: 5 min initial denaturation step at 95 °C, followed by 40 cycles of 95 °C for 20 s, 55 °C for 1 min, and 68 °C for 30 s. Using β-actin as a loading control, results were interpreted by the relative quantity method (ΔΔCt) using β-actin as a loading control as previously described [36]. Primer sequences were: uPA: forward – 5′ GGC AGC AAT GAA CTT CAT CAA GTT CC 3′, reverse – 5′ TAT TTC ACA GTG CTG CCC TCC G 3′; PAI-1:

forward – 5′ AAT CAG ACG GCA GCA CTG TC 3′, reverse – 5′ CTG AAC ATG TCG GTC ATT CC 3′; uPAR: forward – 5′ ACA GGA GCT GCC CTC GCG AC 3′, reverse – 5′ GAG GGG GAT TTC AGG TTA GG 3′; β-Actin: forward – 5′ ATC ATG TTT GAG ACC TTC AA 3′, reverse – 5′ CAT CTC TTG CTC GAA GTC CA 3′.

Immunocytochemistry - 5 x 10^4 cells were plated in 2 well chamber slides (Lab-Tek). After 24 hours cells were washed with PBS and treated with medium containing 1% FBS for 24 hours. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked for 30 minutes with PBS-T containing 1% BSA. After blocking, cells were probed for either Ki67 (Abcam) or the active fragment of caspase 3 (Abcam) for 1 hour at a 1:500 dilution. Primary antibodies were detected by 10 minute incubation with biotinylated ant-rabbit IgG (Vector) followed by 10 minute incubation with streptavidin-HRP (Dako) and 3 minutes of Dab stain (Dako). Cells were counterstained with hematoxylin, mounted, and photomicrographs were obtained with an Olympus BX5W1 connected to an Olympus DB70 microscope digital camera. For each experiment the percentage of positively stained cells was assessed for 10 random 20X fields.

Statistical Analysis - Statistical analysis was performed using a one sample student t test with normal distribution in experimental settings, which are normalized to a theoretical value of 100% or 1 (for quantitative PCR). A two sample student t test with a normal distribution was performed in experimental settings in which control values are not normalized to a theoretical mean of 100%.

Results

MDA-MB-231 cells have a functional plasminogen activator system. To assess the functionality of the plasminogen activator system in MDA-MB-231 cells, we utilized enzymatic inhibition of uPA and PAI-1 in 24 hr transwell invasion assays. Inhibition of uPA with the small molecule inhibitor amiloride and a uPA inhibitory antibody decreased invasion approximately 30% (Figure 5.1). By contrast, inhibition of PAI-1 with an inhibitory antibody increased invasion approximately 48% (Figure 5.1). These results suggest that MDA-MB-231 cells, which express uPAR, uPA, PAI-1 [27] and LRP-1 protein [37], do exhibit a functional plasminogen activator system. Our findings differ from that of Liu *et al.* who reported that inhibition of both uPA and PAI-1 reduced invasion of the lung cancer cell line H292 in a 72 hr transwell assay [38].

Both PI3K and MAPK signaling positively regulate uPA and PAI-1 expression. The PI3K and MAPK signaling pathways are key regulators of proliferation and apoptosis and are often activated in cancer cells. Multiple studies have shown uPA is positively regulated by both PI3K and MAPK signaling. In contrast, MAPK and PI3K signaling has been shown to either positively or negatively regulate PAI-1 expression depending on cell type [39-41]. To determine the influence of these pathways on uPA and PAI-1 expression in the MDA-MB-231 breast cancer cells, cells were treated for 24 hr with the MEK 1/2 inhibitor PD98059 or PI3K inhibitor LY294002, RNA was isolated and reverse transcribed and subjected to quantitative PCR. PI3K inhibition decreased uPA expression at both 10 and 25 μM

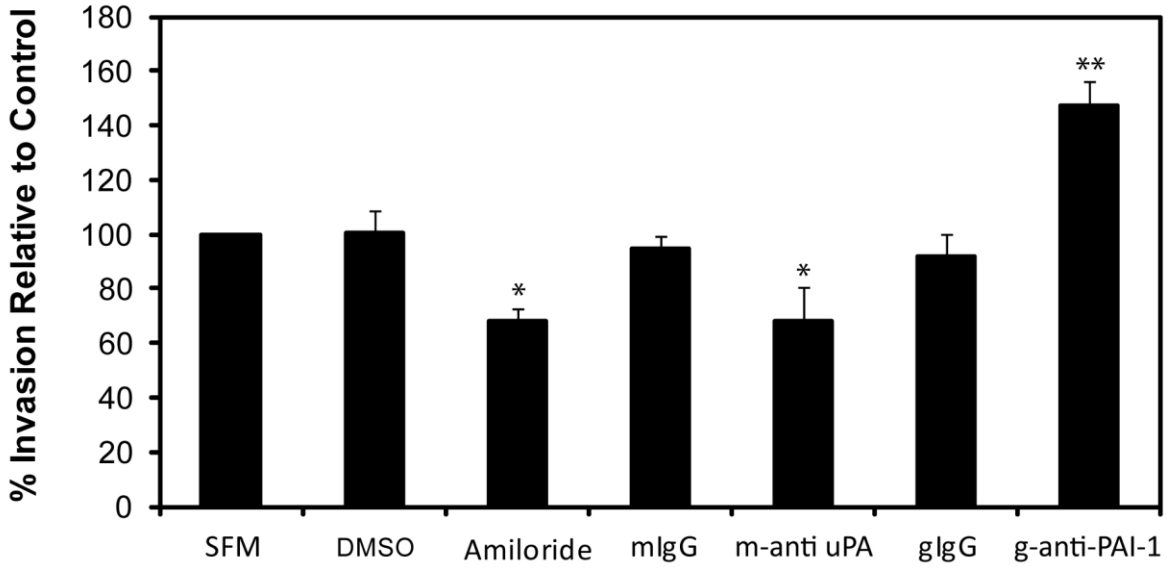

Figure 5.1. Effect of disruption of the plasminogen activator system on invasion and viability of MDA-MB-231 cells. Cells were pre-treated with 0.2 mM amiloride, 20 μg/ml m-anti-uPA, 20 μg/ml g-anti-PAI-1, or corresponding vehicle/control antibodies for 1 hour prior to seeding on matrigel coated inserts. Cells invaded toward 10% FBS media for 24 hours (n=3). Data are expressed as mean +/- SD. *$p<0.05$ and **$p<0.01$, for cells treated with Amiloride, m-anti-uPA and g-anti-PAI-1 relative to DMSO, mIgG, and gIgG, respectively (n=3).

LY294002 (0.55 and 0.63 fold, respectively) and MEK1/2 inhibition decreased uPA expression at both 10 and 25μM PD98059 (0.57 and 0.83 fold, respectively) (Figure 5.2A). Statistically significant inhibition of PAI-1 expression was achieved with 10 μM LY249002 (0.56 fold) and 25 μM PD98059 (0.45 fold) (Figure 5.2B). These results suggest that both MAPK and PI3K signaling positively regulate uPA and PAI-1 expression in the MDA-MB-231 breast cancer cell line.

Establishment and characterization of MDA-MB-231 cells with stable shRNA mediated knockdown of uPA. Multiple studies in cancer cell lines have linked binding of uPA to uPAR to activation of proliferative or anti-apoptotic signaling. To expand upon these findings with the MDA-MB-231 breast cancer cell line, we established MDA-MB-231 cells with stable knockdown of uPA. Cells were infected with a non-targeted lentiviral control (pLKO) or a lentivirus encoding short hairpin RNA directed against uPA mRNA (uPAshRNA#4); stable populations were selected and maintained in puromycin. Immunoblot of concentrated conditioned medium indicate clear knockdown of uPA using the uPAshRNA#4 construct (Figure 5.3A). Interestingly, in addition to uPA knockdown, the uPA shRNA#4 construct also decreased the amount of PAI-1 antigen (Figure 5.3A). The immunoblot results were complemented by quantitative PCR which indicated significant knockdown of uPA (59%, Figure 5.3B, left panel) as well as reduced expression of PAI-1 (40%, Figure 5.3B, right panel) in uPAshRNA#4 cells. The non-targeted pLKO virus did not alter uPA or PAI-1 message levels relative to untreated MDA-MB-231 cells (data not shown). The observed decrease in PAI-1 antigen and mRNA in the uPAshRNA#4 cells was unexpected. However, based on our observations that the uPAshRNA#4 cells have decreased MAPK activity

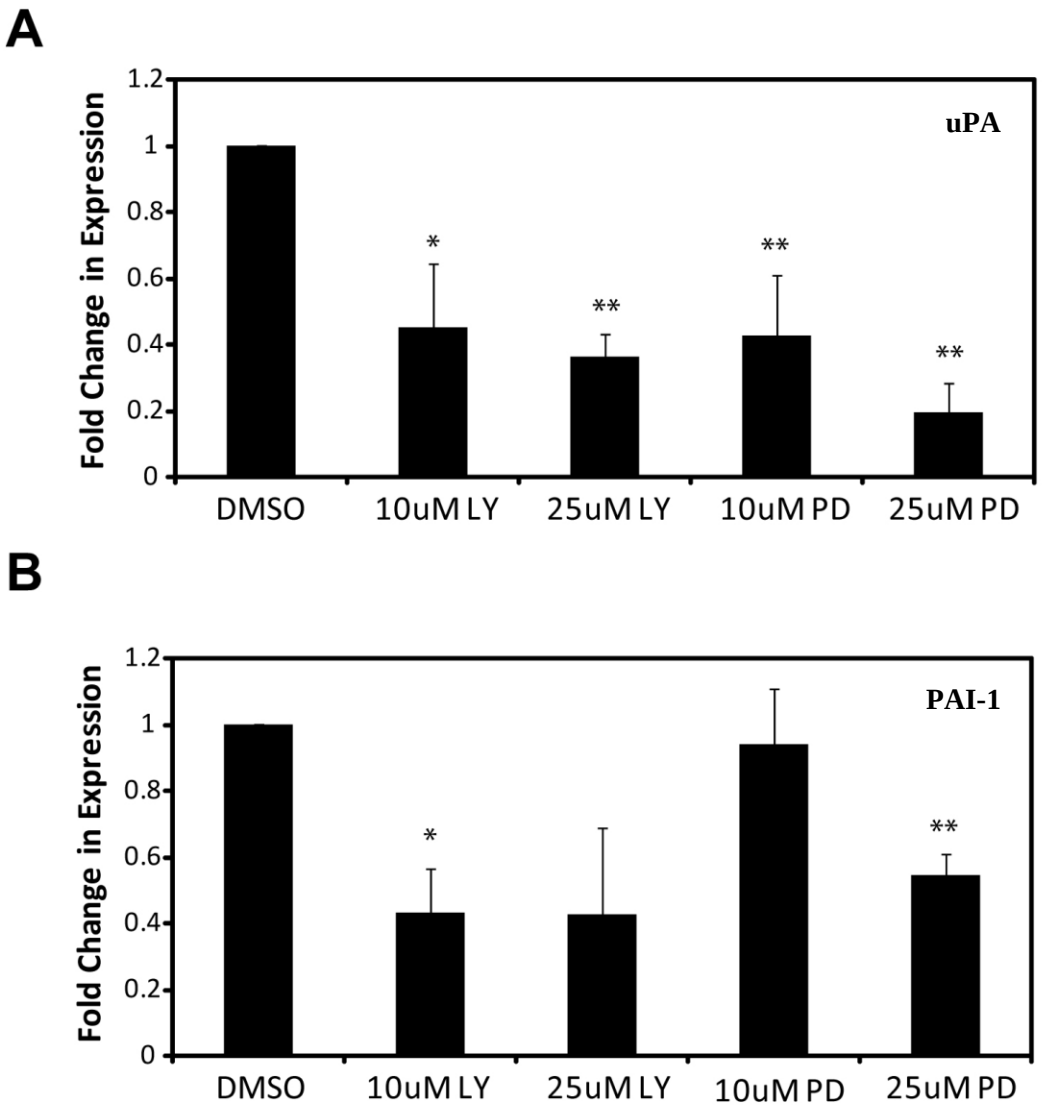

Figure 5.2. PI3K and MAPK signaling modulate uPA and PAI-1 expression. MDA-MB-231 cells were serum starved for 24 hr and then treated for 24 hr with 10 μM or 25 μM PD98059, 10 μM or 25 μM LY249002, or DMSO vehicle control in serum free media. Total RNA was extracted from serum starved cells and reverse transcribed. uPA (upper panel) and PAI-1 (lower panel) expression were examined by quantitative PCR using a SYBR Green probe with β-actin serving as a loading control. Expression was evaluated by the $2^{-\Delta\Delta Ct}$ method relative to DMSO treated cells. Data are expressed as mean +/- SD. *$p<0.05$, **$p<0.01$ (n=3).

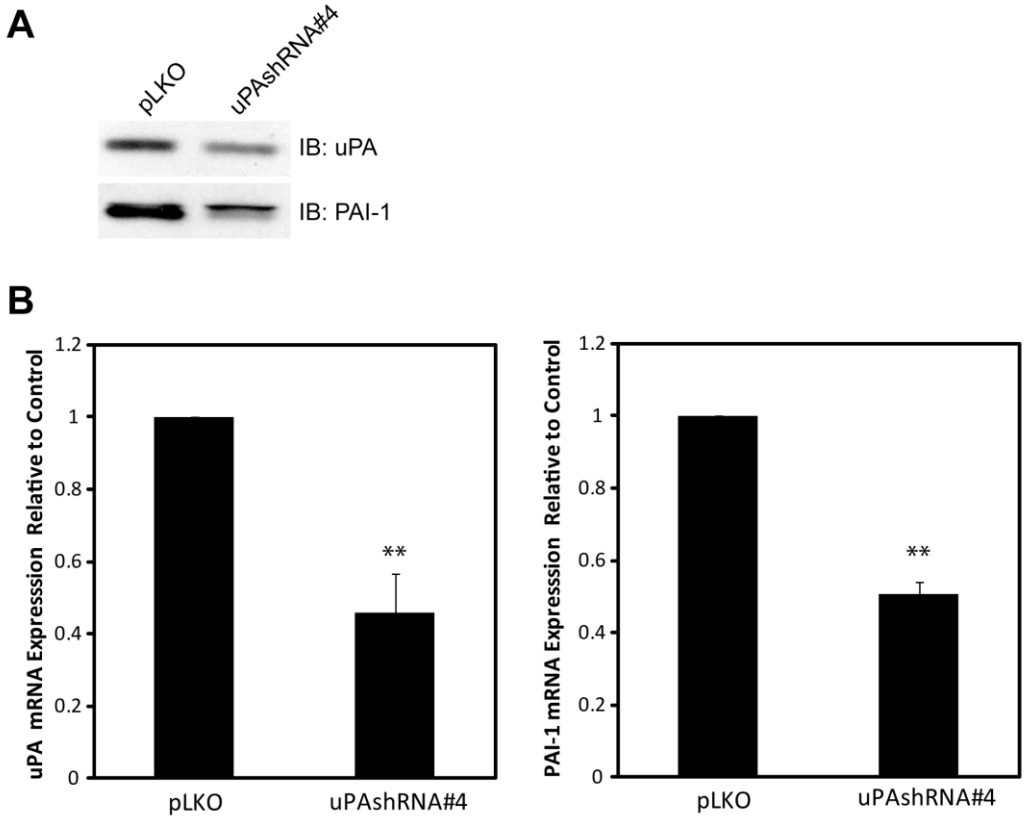

Figure 5.3. Characterization of stable lentiviral mediated knockdown of uPA in MDA-MB-231 cells. (A) Cells expressing non-targeted pLKO control or uPAshRNA#4 lentivirus were serum starved for 24 hours. After 24 hours, conditioned media was collected and concentrated. Equal amount of protein were loaded on polyacrylamide gels and uPA and PAI-1 antigen levels were assayed by immunoblot. (B) Total RNA was extracted from serum starved cells and reverse transcribed. uPA and PAI-1 expression were examined by quantitative PCR using a SYBR Green probe with β-actin serving as a loading control. Expression was evaluated by the $2^{-\Delta\Delta Ct}$ method relative to pLKO cells. Data are expressed as mean +/- SD. (uPA, n=5 and PAI-1 n=3) **p<0.01

(Figure 5.6A, right panel) and that PAI-1 is positively regulated by MAPK signaling in MDA-MB-231 cells (Figure 5.2B), we hypothesize that the diminished PAI-1 levels in the uPAshRNA#4 cells may be a consequence of reduced MAPK signaling.

Using an indirect cell surface uPA activity assay, we observed that the uPAshRNA#4 cells exhibited significantly reduced uPA activity (22%) relative to pLKO control cells (Figure 5.4A). In addition to its catalytic capacity, uPA has also been implicated in adhesion, notably by increasing the affinity of uPAR for the ECM protein vitronectin. Relative to pLKO control cells, uPAshRNA#4 cells showed no change in adhesion to collagen IV, laminin, or fibronectin. However, uPAshRNA#4 cells did exhibit decreased adhesion to vitronectin (Figure 5.4B). Given the combined contributions of uPA to both matrix remodeling and adhesion, we wanted to examine the growth of uPAshRNA#4 in a three dimensional extracellular matrix. When cultured for 6 days in a three dimensional matrix of basement membrane extract, uPAshRNA#4 cells exhibited significantly inhibited colony expansion relative to pLKO control cells (Figure 5.5).

Stable knockdown of uPA in MDA-MB-231 cells reduces MAPK signaling and diminishes cell growth in low serum, but does not diminish cell viability in response to chemotherapeutic agents. Based on previous reports of the capacity of uPA to mediate proliferative and anti-apoptotic signaling, we examined the viability of the uPA stable knockdown cell line relative to the non-targeted pLKO cell line. Growth curves generated by MTT indicated the uPAshRNA#4 cells exhibit diminished growth under low serum conditions (left panel, Figure 5.6A). Supporting the findings of others [31], immunblot of whole cell lysates of uPAshRNA#4 cells also showed reduced ERK phosphorylation relative to pLKO controls, indicative of reduced MAPK signaling (right panel, Figure 5.6A).

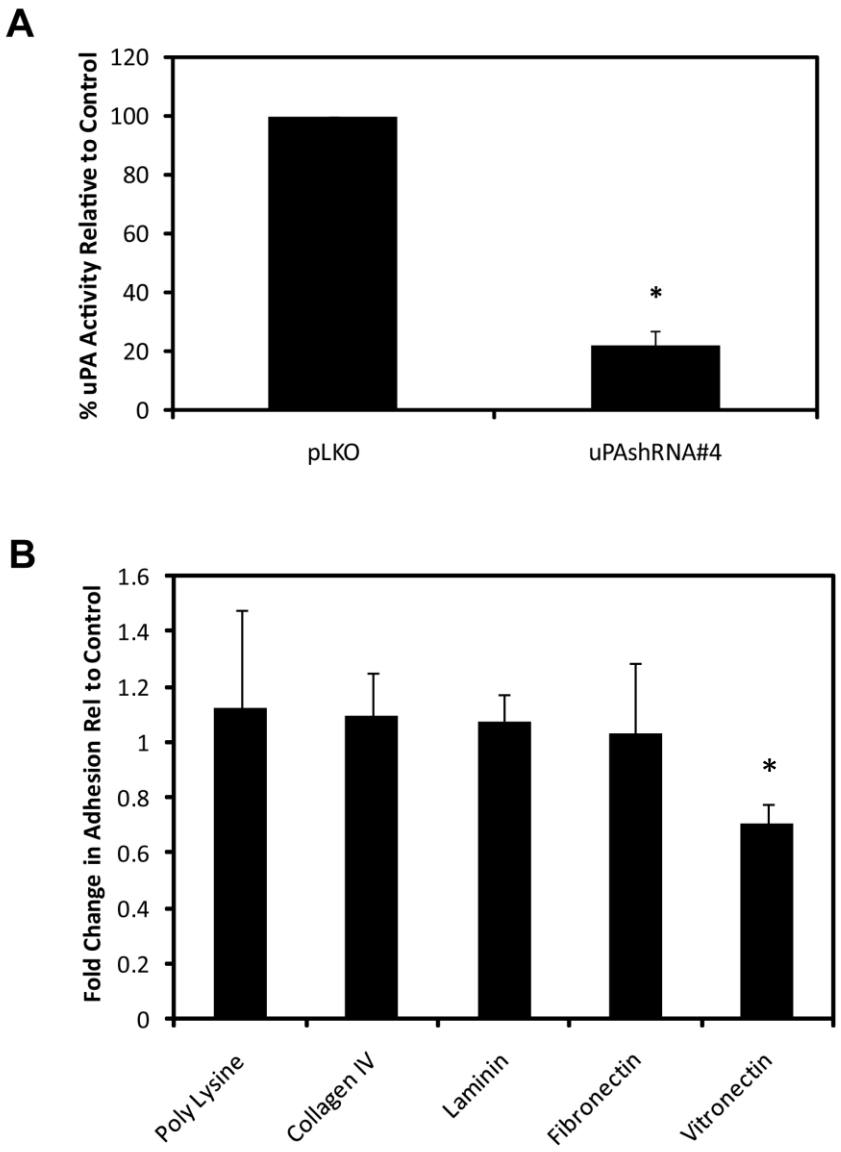

Figure 5.4. Cell-surface associated uPA activity and extracellular matrix adhesion in MDA-MB-231 cells with stable uPA knockdown. (A) uPA activity of pLKO control and uPAshRNA#4 cells was assessed using indirect cell associated uPA activity assays. 20,000 cells were treated with plasminogen for 20 minutes and plasmin activity in the supernatant was assessed by cleavage of a chromogenic substrate. Data are expressed as mean +/- SD (n=3). *$p<0.05$, for uPAshRNA#4 cells relative to pLKO control cells. (B) 25,000 cells pLKO or uPAshRNA#4 cells were plated in serum free media in 96 well plates pre-coated with 0.75 µg collagen IV, laminin, fibronectin, or vitronectin. Cells were allowed to adhere for 1 hr at 37˚C. Non-adherent cells were removed by washing with PBS and the relative amount of adherent cells was assayed by MTT. Data are expressed as mean +/- SD (n=3). *$p<0.05$

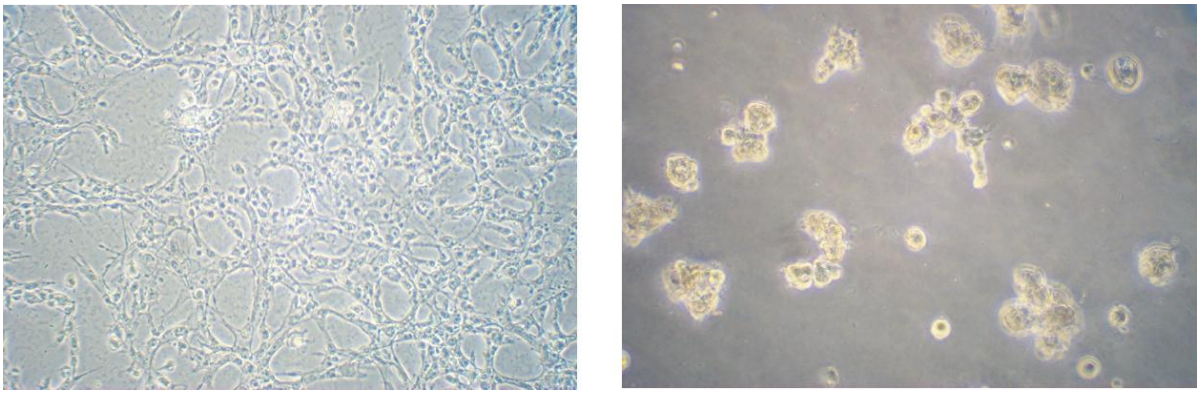

Figure 5.5. Effect of stable uPA knockdown in MDA-MB-231 cells on growth in a three dimensional matrix. 3 x 10^4 pLKO (left panel) or uPAshRNA#4 cells (right panel) were seeded onto plates pre-coated with basement membrane extract (BME) and treated with complete medium containing 10% BME every 2 days. Photomicrographs were obtained after 6 days in culture.

Interestingly, while it has been reported that transient uPA knockdown in MDA-MB-231 cells sensitizes cell to apoptosis under serum free conditions [31] we observed no statistically significant sensitization of uPAshRNA#4 cells in response to apoptosis inducing chemotherapeutic agents paclitaxel, doxorubicin, or cisplatin relative to pLKO control cells (Figure 5.6B)

Inhibition of uPA and PAI-1 activity diminishes cell growth in low serum and growth in a three dimensional matrix. While there is substantial evidence linking uPA binding of uPAR with cell viability and apoptosis, relatively little is known about the contribution of the catalytic activities of uPA and its cognate inhibitor PAI-1 on cancer cell viability. To more closely assess the effect of uPA and PAI-1 inhibition on cell viability, both the MDA-MB-231 and MDA-MB 436 breast cancer cell lines were treated with amiloride, a small molecule uPA active site inhibitor, or PAI-1 inhibitory antibody for a period of 7 days in low serum in a traditional two dimensional cell culture system. Viability curves indicate that both uPA and PAI-1 inhibition have a significant inhibitory effect on the ability of MDA-MB-231 and MDA-MB-436 cells to grow under low serum conditions (Figure 5.7). Given the established role of the PA system in degradation of the extracellular matrix, we next examined if the growth defects observed in low serum conditions translated to inhibited growth in a three dimensional matrix (Figure 5.8). After 6 days in culture in the presence of complete media, we observed that MDA-MB-231 cells treated with amiloride (top right panel, Fig 5.8) exhibited significantly inhibited growth in basement membrane extract relative to control DMSO (top left panel, Figure 5.8). PAI-1 inhibition also yielded a growth defect (bottom right panel, Figure 5.8) relative to control IgG (bottom left panel, Figure 5.8).

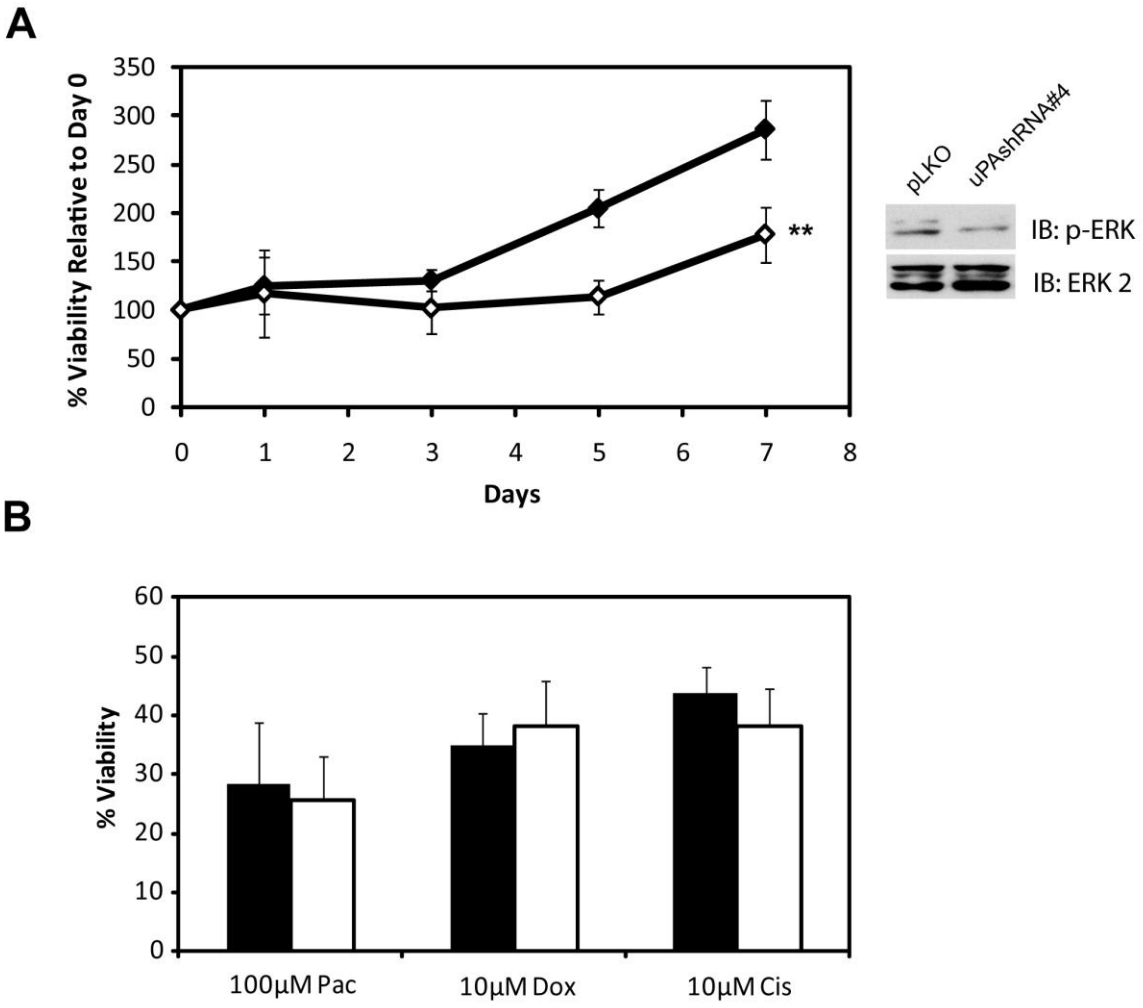

Figure 5.6. Effect of stable uPA knockdown on MDA-MB-231 cells viability in low serum and sensitivity to chemotherapeutic agents. (A) 10,000 cells were treated with 1% FBS media every 2 days and viability was assessed by MTT assay of triplicate samples immediately after initial treatment and at days 1, 2, 3, and 7. Viability is expressed relative to the MTT reading for each cell line at the start of the experiment. Data are expressed as mean +/- SD. For day 7, **p<0.01 for uPAshRNA#4 (◇), relative to pLKO (♦) (n=6). (B) 25,000 pLKO (black bars) and uPAshRNA#4 (white bars) cells in 96 wells plates were treated with complete media supplemented with 100 μM Paclitaxel, 10 μM Doxorubicin, 100 μM Cisplatin, or DMSO vehicle control for 48 hours. Relative viability in response to each chemotherapeutic reagent compared to DMSO alone was assessed by MTT of triplicate samples for each experiment (n=3).

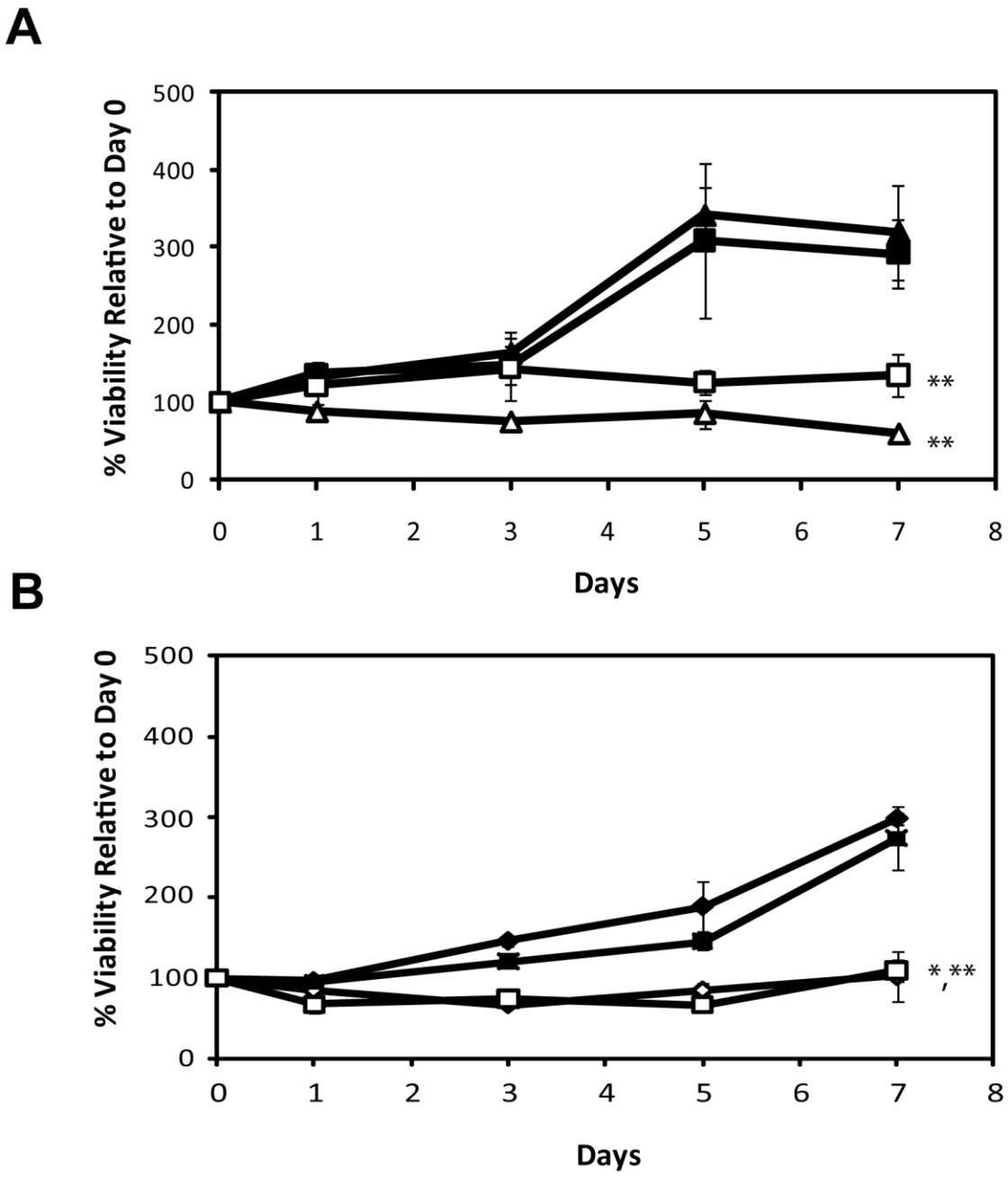

Figure 5.7. Effect of inhibition of uPA and PAI-1 activity on the viability of MDA-MB-231 and MDA-MB-436 cells in low serum. 1 x 10^4 MDA-MB-231 (A) or MDA-MB-436 cells (B) were treated with 1% FBS media supplemented with DMSO (♦), 0.2 mM Amiloride (◊), 20μg/ml gIgGm (■), or 20 μg/ml g-anti-PAI-1 (□) every 2 days and viability was assessed by MTT assay of triplicate samples immediately after initial treatment and at days 1, 2, 3, and 7. Viability is expressed relative to the MTT reading for corresponding wells at the start of the experiment. Data are expressed as mean +/- SD (n=3 for (A), n=2 for (B)). **p<0.01, for cells treated with amiloride relative to DMSO and g-anti-PAI-1 relative to gIgG and *p<0.05, for MDA-MB-436 cells treated with g-anti-PAI-1 relative to gIgG.

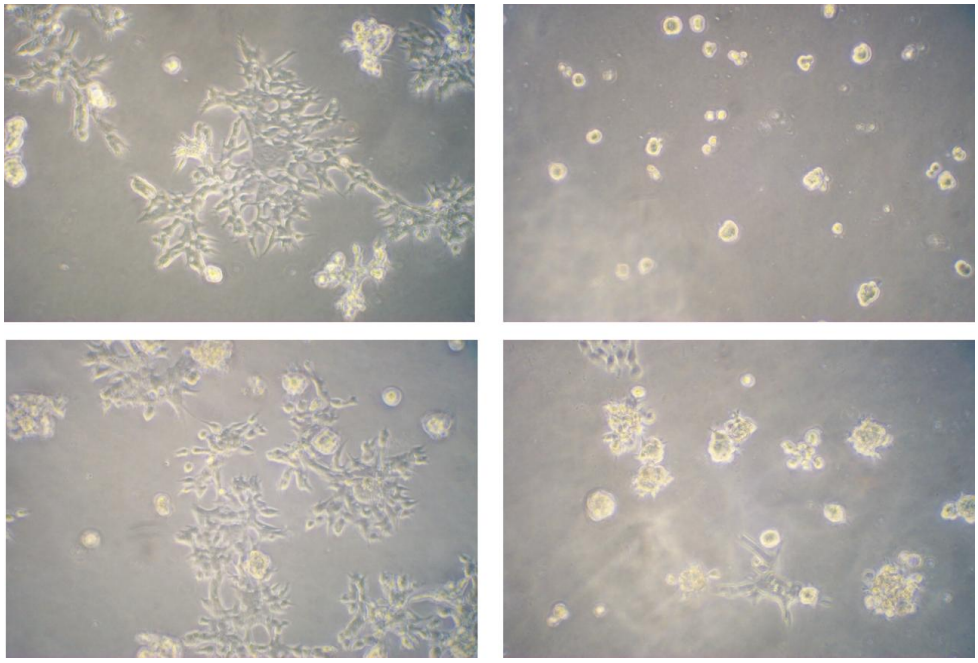

Figure 5.8. Effect of uPA and PAI-1 activity on growth in a three dimensional matrix. 3×10^4 MDA-MB-231 cells were seeded onto plates pre-coated with basement membrane extract (BME) and treated with complete media containing 10% BME supplemented with DMSO (top left panel), 0.2 mM amiloride (top right panel), 20 μg/ml gIgG (bottom left panel), or 20 μg/ml g-anti-PAI-1 (bottom right panel) every 2 days. Photomicrographs were obtained after 6 days in culture.

Based on studies which demonstrate linkages of recognition of uPA and PAI-1 with MAPK and PI3K signaling [42, 43], we hypothesized that the treatments with amiloride and the PAI-1 inhibitory antibody may alter both MAPK and PI3K signaling. By immunoblot for phosphorylated ERK and Akt, we found that uPA and PAI-1 inhibition did yield decreases in both MAPK and PI3K activity for MDA-MB-231 cells. Furthermore, we found that after removing endogenously bound uPA through mild acid wash, treatment of MDA-MB-231 cells with pre-formed uPA-PAI-1 complex activated PI3K more strongly than uPA or PAI-1 alone (Figure 5.9). Together, these results suggest the recognition of uPA-PAI-1 complexes at the cell surface may promote signaling through not only MAPK, which has been previously demonstrated, but also PI3K.

Establishment and characterization of MDA-MB-231 cells with stable shRNA mediated knockdown of PAI-1. Based on our findings using inhibitors of uPA and PAI-1 activity, we next sought to establish MDA-MB-231 cells with stable PAI-1 knockdown. Cells were infected with a non-targeted lentiviral control (pLKO) or lentiviruses encoding short hairpin RNA directed against PAI-1 mRNA (PAI-1shRNA#4); stable populations were selected and maintained in puromycin. Immunoblot of concentrated conditioned media indicates clear knockdown of PAI-1 in the PAI-1shRNA#4 cells relative to pLKO control cells (Figure 5.10A). The immunoblot results were complemented by quantitative PCR that indicated significant knockdown of PAI-1 (85%) in PAI-1shRNA#4 cells (left panel, Figure 5.10B)). The observed increase of uPA antigen in the conditioned media of the PAI-1shRNA#4 cells (Figure 5.10A) is likely a consequence of reduced PAI-1 mediated clearance

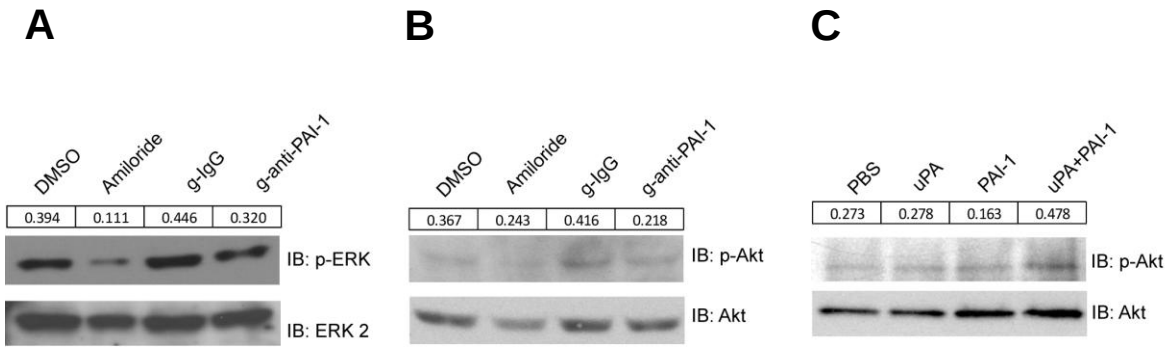

Figure 5.9. Effect of inhibition of uPA and PAI-1 activity on the activity of PI3K and MAPK. Confluent MDA-MB-231 cells were serum starved for 24 hr, washed in PBS, and then treated for 24 hours with serum-free medium supplemented with 0.2 mM Amiloride, 20 μg/ml g-anti-PAI-1, or vehicle/gIgG controls for 24 hr. MAPK and PI3K activity was assessed by immunoblot for p-ERK and total ERK (A) or p-Akt and total Akt (B). (C) Sub-confluent cells were subjected to mild acid wash to remove surface bound uPA and subsequently treated for 5 min with PBS, 10 nM uPA, 20 nM PAI-1, or pre-formed uPA-PAI-1 complex for 5 minutes. PI3K activity PI3K activity was assessed by immunoblot for p-Akt and total Akt on whole cell lysates. Decimals represent fraction of phosphorylated ERK or Akt relative to total as assessed by densitometry.

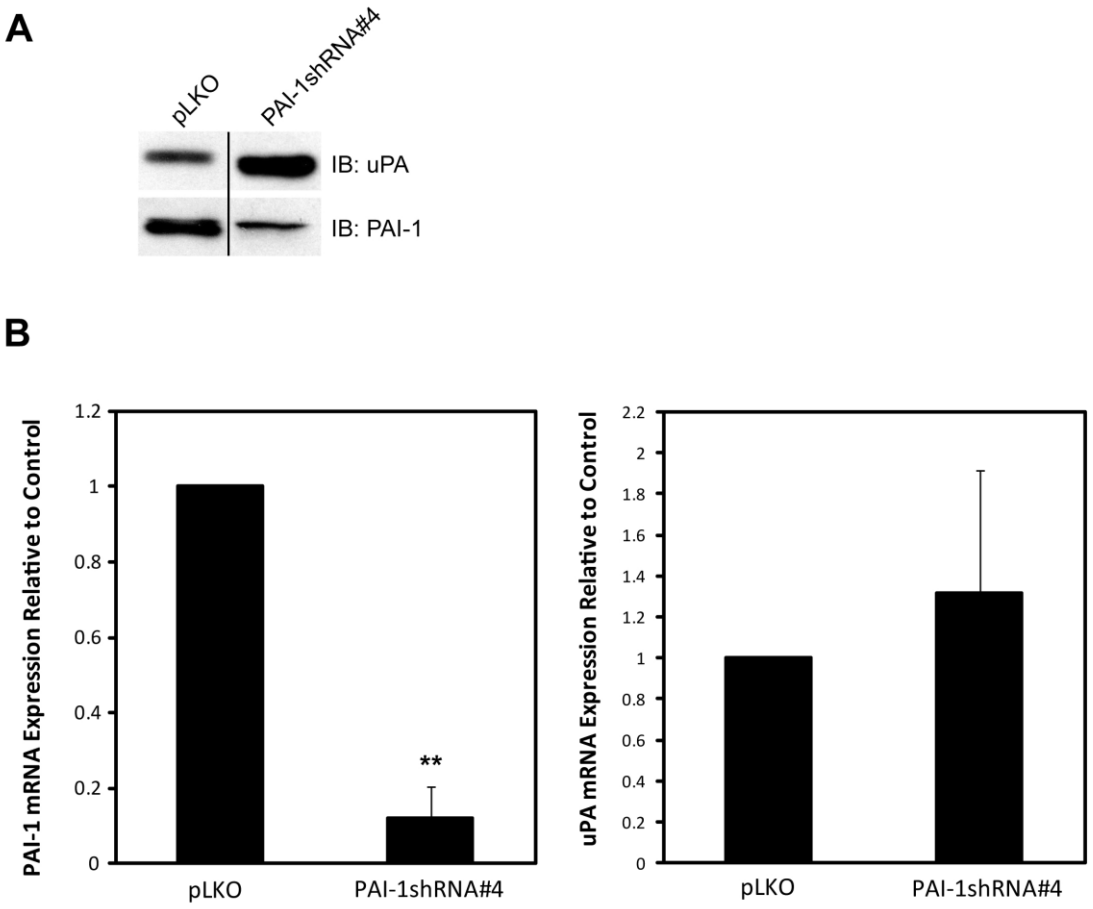

Figure 5.10. Characterization of stable lentiviral mediated knockdown of PAI-1 in MDA-MB-231 cells. (A) Cells expressing non-targeted pLKO control and PAI-1shRNA#4 lentivirus were serum starved for 24 hr. After 24 hr, conditioned media was collected and concentrated. uPA and PAI-1 antigen levels were assayed by immunoblot. (B) Total RNA was extracted from serum starved cells and reverse transcribed. uPA and PAI-1 expression were examined by quantitative PCR using a SYBR Green probe with β-actin serving as a loading control. Expression was evaluated by the $2^{-\Delta\Delta Ct}$ method relative to pLKO cells. Data are expressed as mean +/- SD. **p<0.01, for PAI-1shRNA#4 cells (uPA n=4 and PAI-1 n=6).

of uPA, as uPA expression was not affected by the PAI-1 targeted construct (right panel, Figure 5.10B). Using an indirect cell surface uPA activity assay, we observed that PAI-I knockdown cells exhibited elevated uPA activity (370%) relative to pLKO control cells, as expected (Figure 5.11A). In addition to its capacity to inhibit uPA activity, PAI-1 has been implicated in promoting the de-adhesion of cells from ECM proteins via proposed mechanisms ranging from specifically disrupting uPA/uPAR binding to vitronectin to generally mediating de-adhesion by catalyzing integrin internalization and destruction [15]. PAI-1shRNA#4 cells exhibited significantly enhanced adhesion to collagen IV (1.8 fold), fibronectin (2.1 fold), and vitronectin (1.9 fold) relative to pLKO control cells. Adhesion to laminin was not affected (Figure 5.11B). The observed increased adhesion of PAI-1shRNA#4 cells supports a role for PAI-1 to promote de-adhesion.

Given the pronounced differences in uPA activity and adhesion of the PAI-1shRNA#4 cell relative to pLKO controls, we further examined growth of these cells in a three dimensional matrix. When cultured for 6 days in basement membrane extract PAI-1shRNA#4 cells exhibited slightly reduced expansion as evidenced by a lower cell density (Figure 5.12). This result contrasts with the traditional paradigms of the function of the PA system, which would predict elevated uPA activity would lead to enhanced expansion in a three dimensional matrix.

Stable knockdown of PAI-1 in MDA-MB-231 cells with an alternate construct. Given our novel observations with regard to the relationship of PAI-1 activity and cell viability, before moving forward we generated a secondary cell line with PAI-1 knockdown utilizing an alternate sequence. MDA-MB-231 cells were treated with the alternate PAI-1 directed construct (PAI-1shRNA#5) and again selected in puromycin. The alternate construct clearly

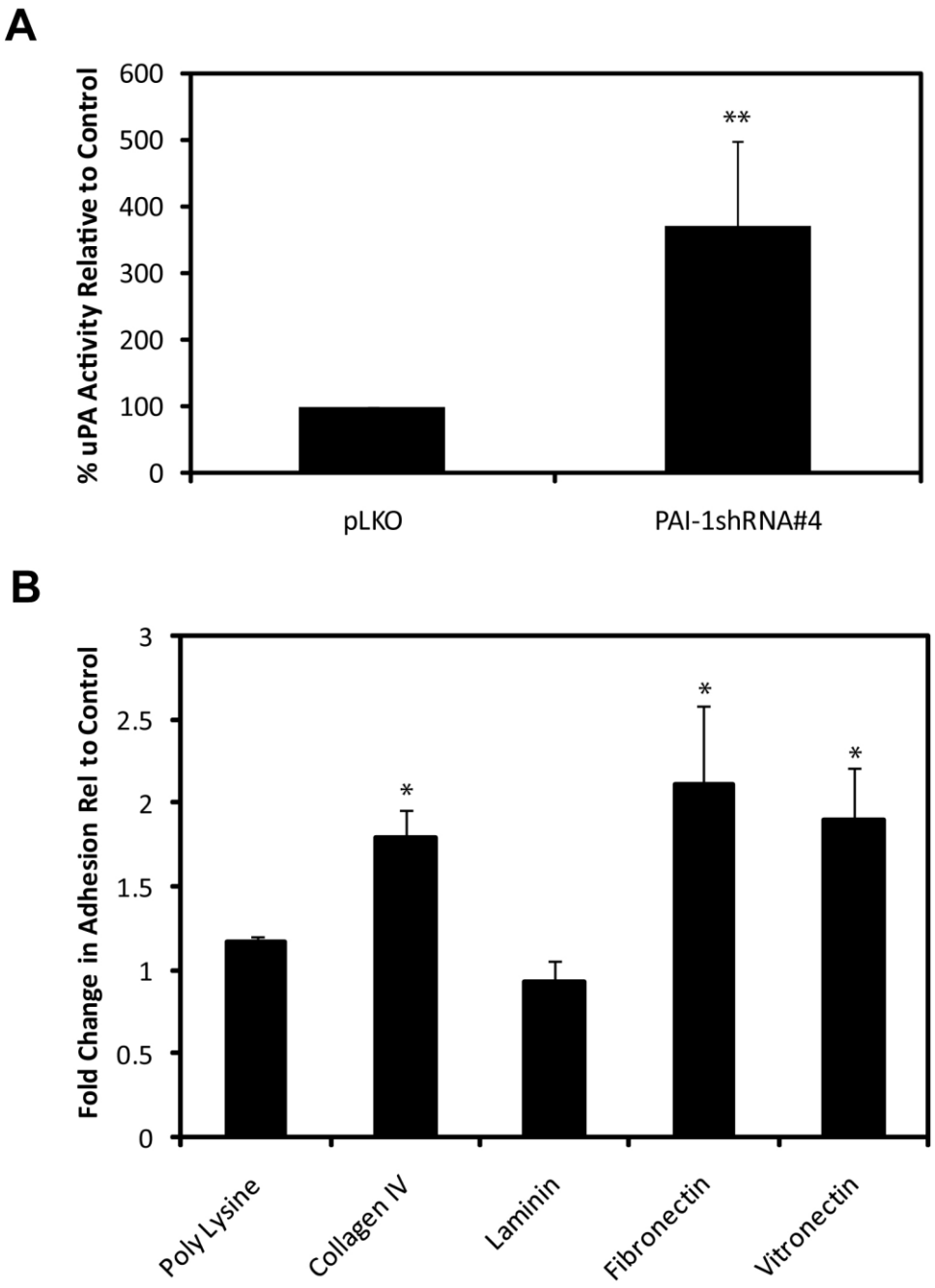

Figure 5.11. Cell-surface associated uPA activity and extracellular matrix adhesion in MDA-MB-231 cells with stable PAI-1 knockdown. (A) uPA activity of pLKO control and PAI-1shRNA#4 cells was evaluated by indirect cell associated uPA activity assays. 2 x 10^4 cells were treated with plasminogen for 20 minutes and plasmin activity in the supernatant was assessed by cleavage of a chromogenic substrate (n=3). *$p<0.05$, **$p<0.01$, for PAI-1shRNA#4 cells relative to pLKO control cells. (B) 2.5 x 10^4 cells pLKO or PAI-1shRNA#4 cells were plated in serum free medium in 96 well plates pre-coated with 0.75 µg collagen IV, laminin, fibronectin, or vitronectin. Cells were allowed to adhere for 1 hr at 37˚C. Non-adherent cells were removed by washing with PBS and the relative amount of adherent cells was assayed by MTT.

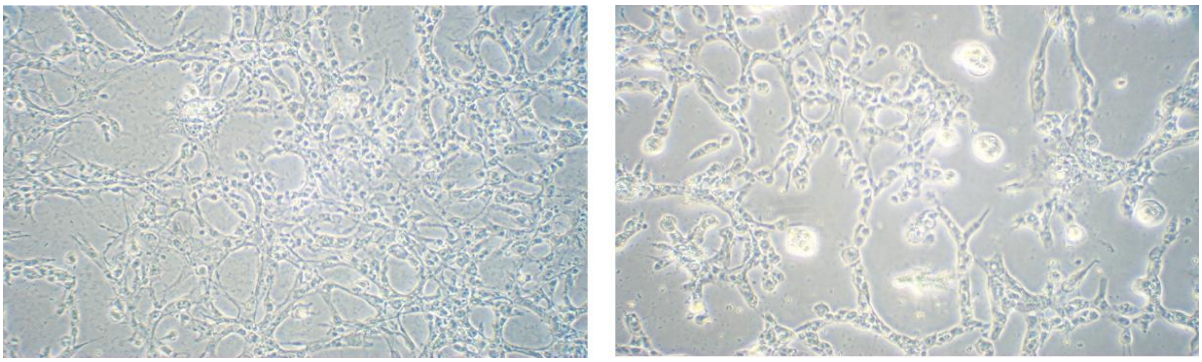

Figure 5.12. Effect of stable PAI-1 knockdown in MDA-MB-231 cells on growth in a three dimensional matrix. 3 x 10^4 pLKO (left panel) or PAI-1shRNA#4 (right panel) cells were seeded onto plates pre-coated with basement membrane extract (BME) and treated with complete media containing 10% BME every 2 days. Photomicrographs were obtained after 6 days in culture.

reduced PAI-1 antigen in conditioned media and decreased PAI-1 mRNA by 70% (Figure 5.13), without affecting uPA mRNA (data not shown).

Stable knockdown of PAI-1 in MDA-MB-231 cells diminishes cell growth in low serum and alters MAPK and PI3K signaling. Based on our findings with uPA and PAI-1 inhibition, we examined the viability of the two PAI-1 stable knockdown cell lines relative to the non-targeted pLKO cell line. Growth curves generated by MTT indicated both PAI-1shRNA#4 and PAI-1shRNA#5 cells exhibit diminished growth under low serum conditions (Figure 5.14). When MAPK and PI3K activity was assessed by immunoblot of phosphorylated ERK and Akt in the cell lines, both PAI-1shRNA#4 and PAI-1shRNA#5 knockdown cell lines exhibited reduced MAPK (Figure 5.15A) and PI3K activity (Figure 5.15B). Densitometric analysis of immunoblots for p-ERK *versus* total ERK and p-Akt *versus* total Akt showed that PAI-1shRNA#4 and PAI-1shRNA#5 cells exhibit an approximate 59% and 70% reduction in p-ERK signal and 80% and 75% reduction in p-Akt signal, respectively, relative to pLKO control cells. This data shows that MDA-MB-231 cells with stable PAI-1 knockdown have reduced overall viability relative to control cells under low serum conditions and this difference may be due to a combined reduction in MAPK and PI3K signaling.

MDA-MB-231 cells with stable PAI-1 knockdown are sensitized to apoptosis but do not have reduced proliferative capacity. The growth curves show that MDA-MB-231 cells with PAI-1 knockdown exhibit reduced growth under low serum conditions. In low serum conditions, the overall phenotype could be attributed to either a defect in the proliferative capacity of the cells or increased cell death due to apoptosis. To address which of these

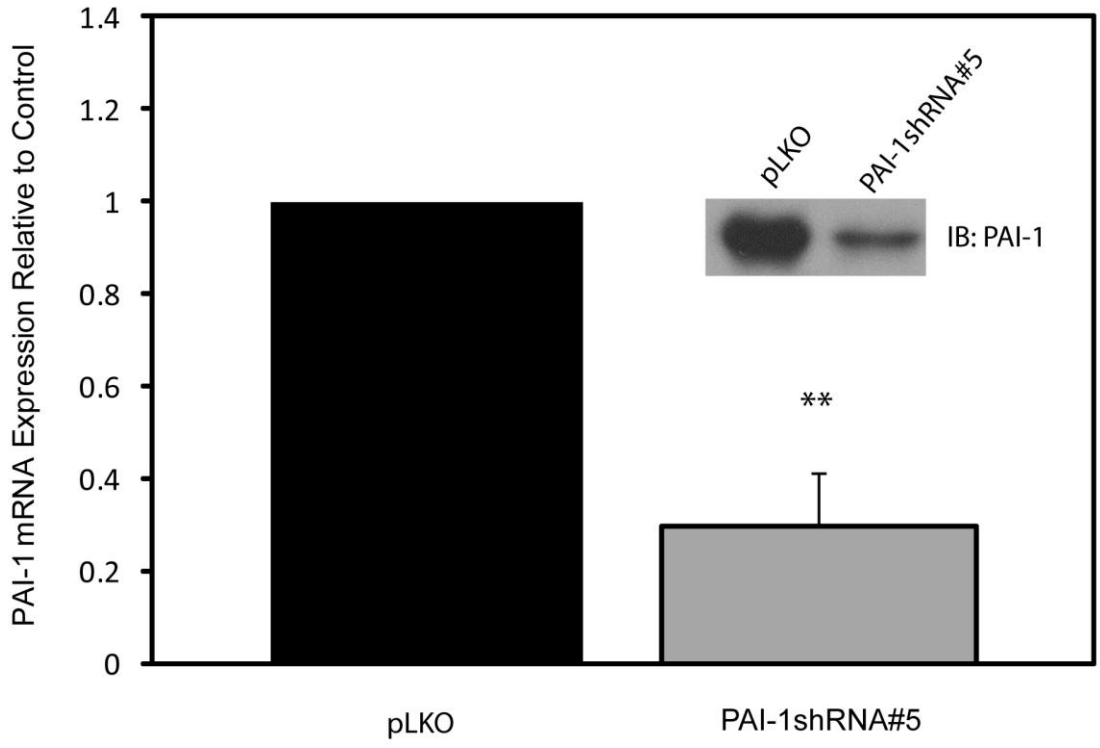

Figure 5.13. Characterization of MDA-MB-231 cells expressing PAI-1shRNA#5. Total RNA was extracted from serum starved cells and reverse transcribed. PAI-1 expression was examined by quantitative PCR using a SYBR Green probe with β-actin serving as a loading control. Expression was evaluated by the $2^{-\Delta\Delta Ct}$ method for PAI-1shRNA#5 relative to pLKO cells. Data are expressed as mean +/- SD (n=3). **p<0.01. Inset. pLKO control and PAI-1shRNA#5 cells were serum starved for 24 hr. After 24 hr, conditioned media was collected and concentrated. PAI-1 antigen levels were assayed by immunoblot.

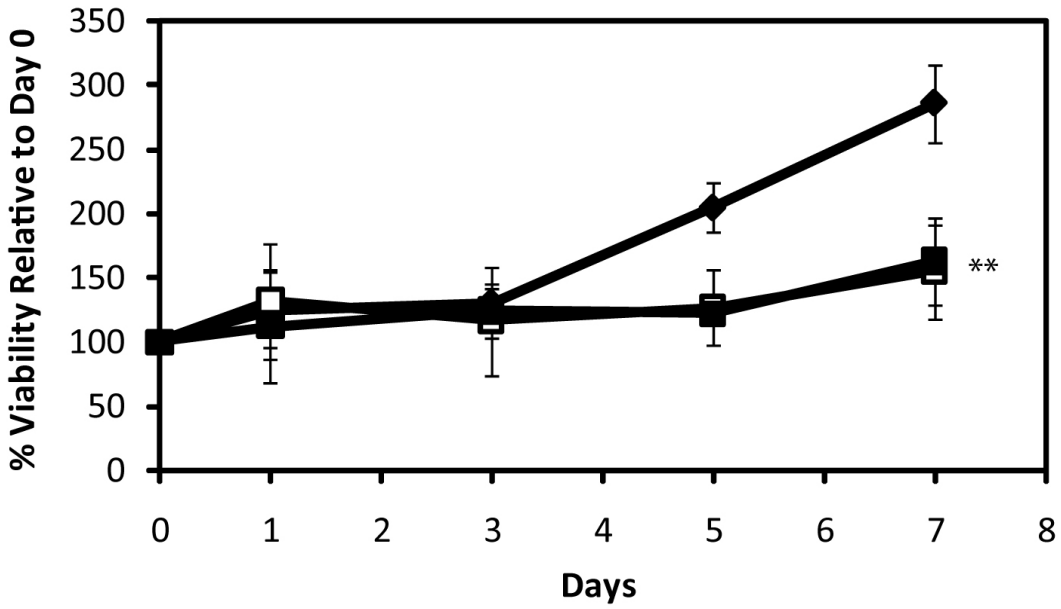

Figure 5.14. Effect of stable PAI-1 knockdown in MDA-MB-231 cells on viability in low serum. 1 x 10^4 cells were treated with 1% FBS media every 2 days and viability was assessed by MTT assay of triplicate samples immediately after initial treatment and at days 1, 2, 3, and 7. Viability is expressed relative to the MTT reading for each cell line at the start of the experiment. Data are expressed as mean +/- SD. For day 7, **p<0.01 for PAI-1shRNA#4 (□, n=4), and PAI-1shRNA#5 (■, n=3), relative to pLKO (♦, n=6).

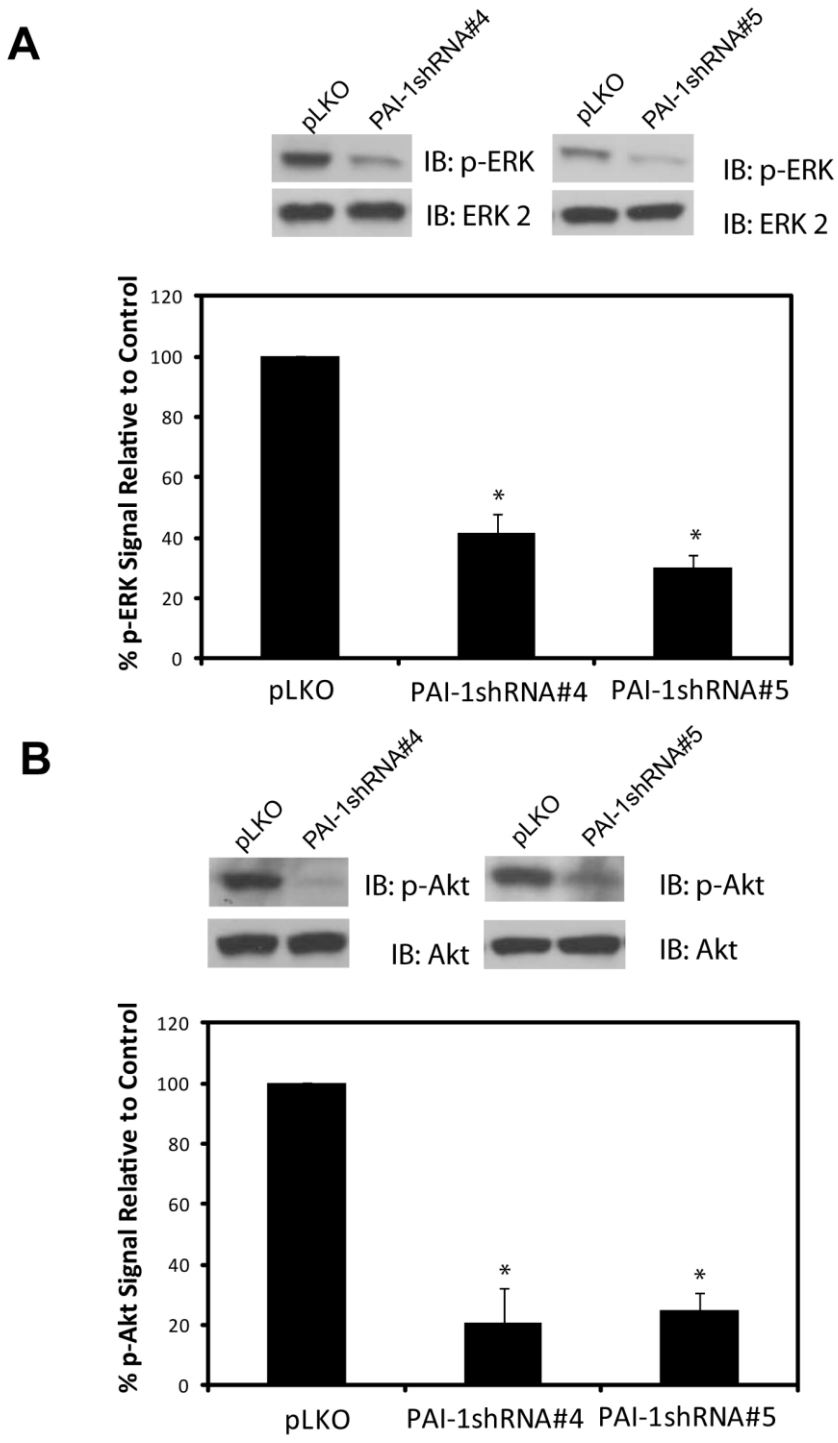

Figure 5.15. Effect of stable PAI-1 knockdown on PI3K and MAPK activity. Confluent cells were serum starved for 24 hr and MAPK and PI3K activity was assessed by immunoblot of whole cell lysates for p-ERK and total ERK (A) or p-Akt and total Akt (B). Percentage p-ERK/ERK and p-Akt/Akt signal in PAI-1shRNA#4 and PAI-1shRNA#5 relative to pLKO control cells was further assessed by densitometry of immunoblots from two independent experiments. Data are expressed as mean +/- SD (n=2). *$p<0.05$.

phenomena contributes most to the reduced growth of PAI-1shRNA#4 and PAI-1shRNA#5 cells relative to the pLKO control, cells were treated for 24 hours in low serum conditions and then stained for either the proliferation marker Ki67 or the active fragment of Caspase-3, a key initiator of the apoptotic proteolytic cascade. When compared to both wild type MDA-MB-231 and pLKO cells, the PAI-1 knockdown cells exhibited no decrease in Ki67 staining (Figure 5.16A). Given that no differential was observed in the four cell lines, as a control, late passage human umbilical vein endothelial cells (HUVECs) were stained for Ki67. Only 43% of these late passage HUVEC stained positive for Ki67 (data not shown). However, both PAI-1shRNA#4 and PAI-1shRNA#5 cell lines had increased active Caspase-3 staining (38% and 34%, respectively) relative to both wild type MDA-MB-231 cells and pLKO cells (8% and 9%, respectively) (Figure 5.16B). These results suggest that the growth defect observed under low serum conditions for the PAI-1 knockdown cells is likely a consequence of increased cell death due to apoptosis.

PAI-1 knockdown sensitizes MDA-MB-231 cells to apoptosis-inducing chemotherapeutic agents. To further examine our hypothesis that PAI-1 knockdown sensitizes cells to apoptosis, pLKO, PAI-1shRNA#4, and PAI-1shRNA#5 cells were treated with the microtubule stabilizing agent paclitaxel, the DNA intercalating agent doxorubicin, and DNA crosslinking agent cisplatin for 48 hours in complete media. As assayed by MTT, there was significantly more cell death in response to all three agents in the PAI-1 knockdown cell lines relative to pLKO control cells, except for the response of PAI-1shRNA#5 cells to cisplatin which was not statistically significant (Figure 5.17A). Wild type MDA-MB-231 and pLKO cells did not exhibit differential sensitivity to the chemotherapeutic drugs (data not shown). pLKO and PAI-1shRNA#4 cells were also

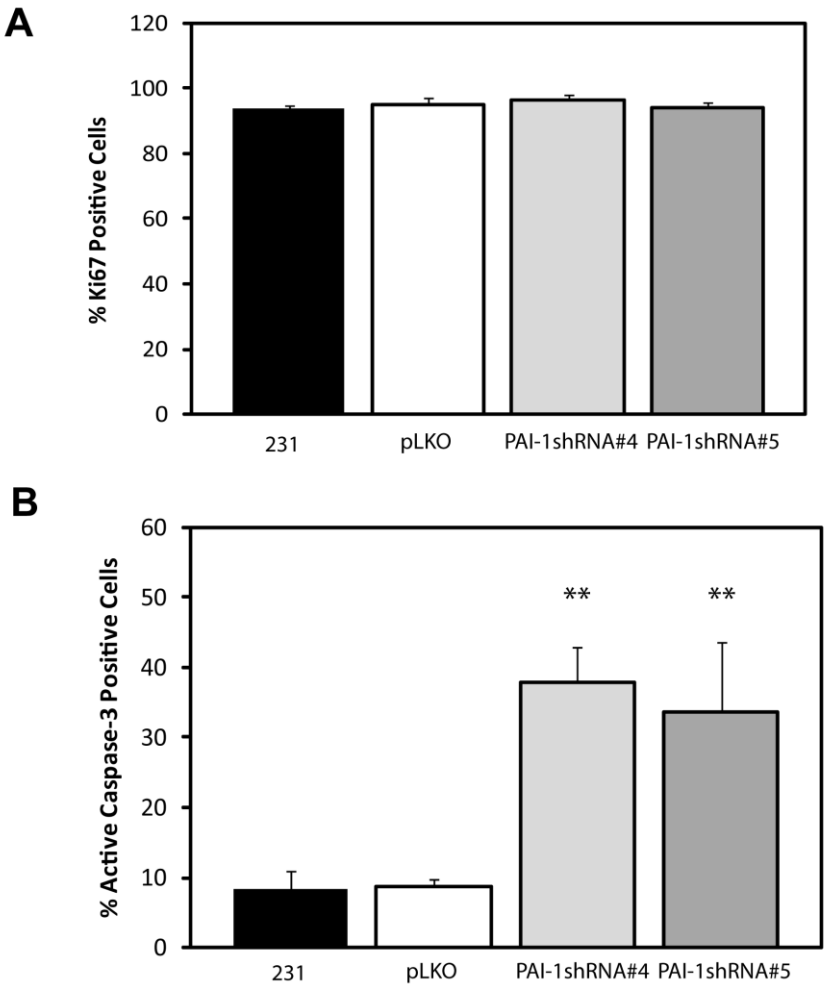

Figure 5.16. Assessment of basal proliferative capacity and apoptosis in MDA-MB-231 cells with stable PAI-1 knockdown. 50,000 cells were plated in 2 well chamber slides. 24 hours post plating cells were treated with 1%FBS media for 24 hours, fixed in 4% paraformaldehyde, and stained for Ki67 (A) to assess proliferative capacity or active Caspase-3 (B) to assess apoptosis. For each experiment, percent positivity was determined by counting 10 random fields at 20X magnification. Data are expressed as mean +/- SD (n=3). **p<0.01, for PAIshRNA#4 (gray bars) and PAI-1shRNA#5 (dark gray bars) relative to wild type MDA-MB-231 cells (black bars) and well as pLKO control cells (white bars).

cultured in a three dimensional matrix of basement membrane extract in the presence of paclitaxel, doxorubicin, and cisplatin for 6 days. Relative to the pLKO cells in response to paclitaxel and cisplatin, there was significantly more death of the PAI-1shRNA#4 cells, evidenced by rounding and detachment from the basal culture surface. In response to doxorubicin, while some pLKO cells remained viable in culture, after 6 days in culture only cellular fragments remained of the PAI-1shRNA#4 cells (Figure 5.17B). Collectively, these results indicate that stable knockdown of PAI-1 significantly sensitizes MDA-MB-231 cells to chemotherapeutic agents.

Inhibition of PAI-1 activity sensitizes MDA-MB-436 cells to apoptosis inducing chemotherapeutic agents. As discussed previously, MDA-MB-436 cells displayed growth inhibition in low serum conditions similar to the MDA-MB-231 cells. To further assess the role of PAI-1 in sensitivity to chemotherapeutic agents these cells were also treated with paclitaxel, doxorubicin, and cisplatin in the presence of control goat IgG or PAI-1 inhibitory antibody. The MDA-MB-436 cells were more sensitive to these agents that the MDA-MB-231 cells (data not shown). However, treatment with all three agents at a lower concentration (1 μM) revealed that inhibition of PAI-1 activity significantly increased their sensitivity to paclitaxel, doxorubicin, and cisplatin relative to control goat IgG (Figure 5.18).

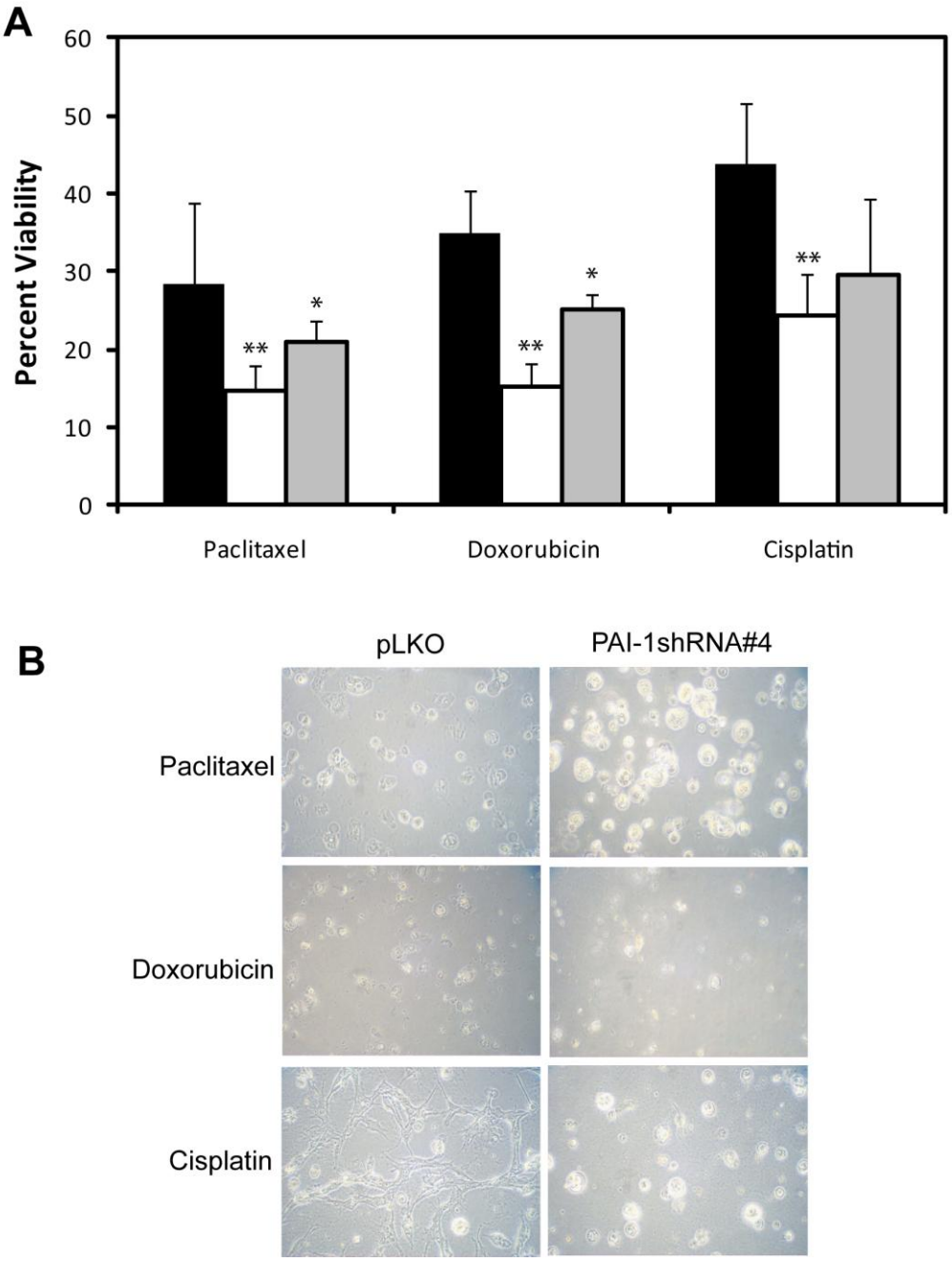

Figure 5.17. Chemotherapeutic sensitivity of MDA-MB-231 cells with stable PAI-1 knockdown. (A) 2.5 x 10^4 pLKO (black bars), PAI-1shRNA#4 (white bars), and PAI-1shRNA#5 (gray bars) cells in 96 wells plates were treated with complete media supplemented with 100 μM Paclitaxel, 10 μM Doxorubicin, 100 μM Cisplatin, or DMSO vehicle control for 48 hr. Relative viability in response to each chemotherapeutic reagent compared to DMSO alone was assessed by MTT of triplicate samples for each experiment (n=3). *$p<0.05$, **$p<0.05$, for PAI-1shRNA#4 and PAI-1shRNA#5 relative to pLKO control cells. (B) 30,000 pLKO and PAI-1shRNA#4 cells were seeded onto plates pre-coated with basement membrane extract (BME) and treated with complete media containing 10% BME supplemented with 100 μM Paclitaxel, 10 μM Doxorubicin, or 100 μM Cisplatin every 2 days. Photomicrographs were obtained after 6 days in culture.

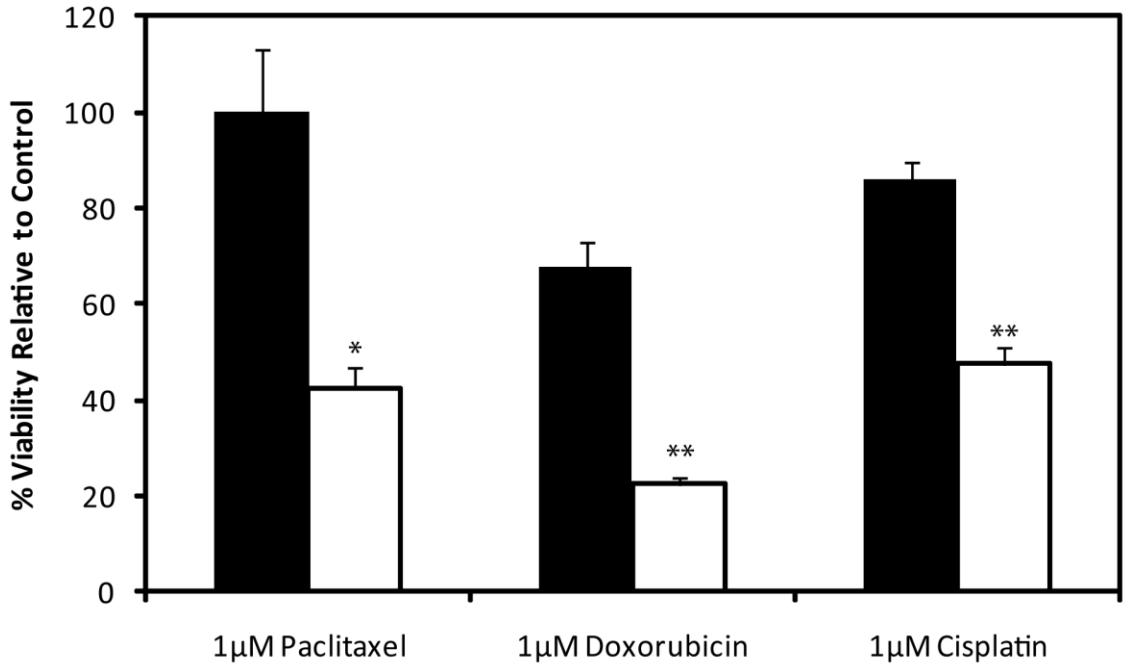

Figure 5.18. Sensitization of MDA-MB-436 cells to chemotherapeutic agents by PAI-1 activity inhibition. 25,000 MDA-MB-436 cells in 96 wells plates were treated with complete media supplemented with 1µM Paclitaxel, 1µM Doxorubicin, 1µM Cisplatin, or DMSO vehicle control with gIgG control antibody (black bars) or goat PAI-1 inhibitory antibody(white bars) for 48 hours. Relative viability in response to each chemotherapeutic reagent compared to DMSO alone was assessed by MTT of triplicate samples for each experiment. Data are expressed as mean +/- SD (n=2). *$p<0.05$, **$p<0.01$.

Discussion

The plasminogen activator system has proven to play a complex role in tumor biology related to angiogenesis, tumor cell motility, and viability. However, likely due to the complex interplay of uPAR, the protease uPA, the serpin PAI-1, and the LRP-1 co-receptor, as well the interplay between this system with integrins and adhesion to the ECM, conflicting results emerge depending on experimental context. Notably, most of the conflicting data centers on the biological activity of the serpin, PAI-1. For example, with regard to angiogenesis, Soff et al. showed that expression of PAI-1 cDNA in prostate carcinoma cells inhibits angiogenesis [23], while studies by Bajou et al. showed that absence of PAI-1 prevents tumor vascularization [22]. Furthermore, conflicting results also have been reported regarding the role of PAI-1 in tumor cell motility. In cell motility assays on vitronectin, Kjoller et al. reported PAI-1 inhibits motility of epidermoid carcinoma Hep-2 cells [44] whereas Palmieri et al. reported PAI-1 promotes motility [45]. In this report, using the MDA-MB-231 breast cancer cell line, we utilized the transwell invasion assay that incorporates motility and proteolytic capacity as a surrogate for a metastatic phenotype. In this experimental context, inhibition of uPA activity leads to reduced invasion whereas PAI-1 inhibition promotes invasion. These findings correlate with the traditional paradigm of the function of the PA system in cancer cell metastasis.

Outside of the influence of PAI-1 in angiogenesis and cell motility, an emerging complexity in the field is the influence of PAI-1 on tumor cell viability. There are numerous reports suggesting that uPA/uPAR activates signaling pathways associated with proliferation and protection from apoptosis in cancer cells [46-50]. This signaling capacity of uPA/uPAR is important given the observed up-regulation of uPA and uPAR in multiple types of cancer

[14, 51-53]. It is possible that our degree of uPA knockdown was not sufficient to sensitize cells to apoptosis through reduced MAPK signaling.

What remains unclear is how PAI-1, in the presence of uPA, modulates signaling associated with proliferation and/or apoptosis. This question is especially relevant to breast cancer, as elevated levels of both uPA and PAI-1 antigen are strongly associated with poor prognosis [21]. One might expect PAI-1 would logically inhibit uPA/uPAR mediated proliferative and/or anti-apoptotic signaling and therefore lead to reduced viability of a cancer cell. However, there is growing evidence supported by our findings here, that PAI-1 can instead actually promote cancer cell viability.

Webb et al. reported that in MCF-7 breast cancer cells, recognition of purified uPA-PAI-1 complex at the cell surface stabilized ERK phosphorylation and was associated with increased cell viability in serum free medium relative to treatment with uPA or PAI-1 alone [42]. In this report, we show that in the inverse experiment, disruption of uPA-PAI-1 complex formation with both the uPA inhibitor amiloride and an antibody directed at the reactive center loop of PAI-1 results in reduced MAPK activity, reduced viability in serum free media, and reduced growth of cells in low serum conditions over a 7-day period. Furthermore, we report that in addition to ERK phosphorylation, inhibiting uPA-PAI-1 complex formation was also associated with decreased Akt phosphorylation whereas treatment of cells with pre-formed uPA-PAI-1 complex resulted in increased Akt phosphorylation. Phosphorylation of Akt is a key indicator of activation of the anti-apoptotic PI3K pathway and suggests the effects of uPA-PAI-1 complex formation and recognition at the cell surface are related to protection from apoptosis.

We also aimed to evaluate the phenotype of cells cultured in a three dimensional matrix with uPA and PAI-1 inhibition because a combination of cell proliferation, matrix adhesion, and proteolytic activity all contribute to colony expansion. In three-dimensional culture, inhibition of uPA activity with amiloride significantly inhibited colony expansion in matrigel likely as a combined consequence of decreased proliferative/anti-apoptotic signaling and decreased capacity for matrix degradation. Interestingly, despite the potential for increased capacity for matrix degradation, antibody-mediated inhibition of PAI-1 activity also partially decreased expansion in a three-dimensional culture, again likely a consequence of reduced proliferative capacity and/or increased sensitivity to apoptosis.

Given the limitations of enzymatic inhibition, we aimed to establish MDA-MB-231 derivative cell lines with stable shRNA mediated knockdown of either uPA or PAI-1 in parallel to examine the resulting proliferative/anti-apoptotic phenotype. As expected, relative to the finding of Ma et al [31], cells with stable knockdown of uPA exhibited reduced ERK phosphorylation. Ma et al further suggested that uPA knockdown sensitizes MDA-MB-231 cells to apoptosis. However, we did not observe sensitization to apoptosis inducing chemotherapeutic agents. This discrepancy may be a result of knockdown strategy as the previous study utilized transient knockdown whereas we generated a stable population.

Intriguingly, uPA knockdown unexpectedly led to a decrease in PAI-1 mRNA expression. We hypothesize the reduced PAI-1 expression is a consequence of reduced ERK phosphorylation as PAI-1 expression is positively regulated by MAPK activity in the parental MDA-MB-231 cells. As observed with uPA enzymatic inhibition, uPA knockdown significantly inhibited growth of cells in low serum and the expansion of cells in three-

dimensional culture. However, we did not observe sensitization to apoptosis inducing chemotherapeutic agents.

Cells with stable knockdown of PAI-1 exhibited an inhibited ability to grow in low serum conditions similar to that observed with uPA knockdown as well as a decrease in expansion in a three dimensional matrix. Interestingly, PAI-1 knockdown resulted in a pronounced reduction of both ERK and Akt phosphorylation. This finding correlates with the recent results of Romer *et al* who demonstrated fibrosarcoma cell lines derived from PAI-1 knockout mice, as well as with wild type fibrosarcoma lines treated with PAI-1 RNAi, exhibit reduced Akt phosphorylation relative to controls [54].

Because cell growth in low serum conditions represents a balance of proliferation *versus* apoptosis, we wanted to assess more closely the predominant process affecting growth in low serum conditions secondary to PAI-1 knockdown. Notably, PAI-1 knockdown did not significantly inhibit expression of Ki67, a marker of cell cycling [55]. By contrast, PAI-1 knockdown did significantly increase activation of the cysteine protease Caspase-3, a key initiator of apoptosis [56]. Taken together with the decreased phosphorylation of both ERK and Akt, our results suggest PAI-1 can protect cells from spontaneous apoptosis. Based on *in vitro* activity and binding assays, it has been previously suggested that PAI-1, which is a serine protease inhibitor, may protect cells from apoptosis intracellularly via direct inhibition of Caspase-3 cysteine protease activity in vascular smooth muscle cells [57]. However, the observed increase activation of Caspase-3 in this report suggests the anti-apoptotic effects of PAI-1 are upstream of direct inhibition of Caspase-3. The observed viability defect secondary to antibody mediated PAI-1 inhibition in low serum conditions for both MDA-MB-231 and MDA-MB-436 breast cancer cells suggests PAI-1 exerts its anti-apoptotic

effects by initiating signaling extracellularly, possibly through the LRP-1 co-receptor that has been demonstrated to exert anti-apoptotic signaling capacity through PI3K [58-59]. It is also interesting that MAPK and PI3K signaling which are activated by PAI-1 at the cell surface, also positively regulate expression of uPA and PAI-1, potentially creating a feedback loop. Figure 5.19 diagrams our working model of how recognition of PAI-1, and specifically, uPA-PAI-1 complexes at the cell surface may initiate signaling through LRP-1 and also positively regulate uPA and PAI-1 expression.

Given the observed increased sensitivity to spontaneous apoptosis, we further evaluated the sensitivity of cells with PAI-1 knockdown to apoptosis-inducing agents. Endogenous PAI-1 was previously shown to be protective against etoposide-induced apoptosis in murine fibrosarcoma cell lines [54, 60]. In addition, purified PAI-1 has been demonstrated to protect the human prostate cancer cell line PC-3 and the human promeylocytic leukemia cell line HL-60 from CPT-induced apoptosis but pre-formed uPA-PAI-1 complex did not [61]. Here we report that PAI-1 knockdown in MDA-MB-231 cells, which also express uPA, significantly sensitizes the cells to apoptosis-inducing agents paclitaxel, doxorubicin, and cisplatin. We also demonstrate that MDA-MB-436 breast cancer cells can be sensitized to apoptosis inducing chemotherapeutic drugs with a PAI-1 inhibitory antibody. It is notable that these three agents work through distinct mechanisms. While paclitaxel is a microtubule stabilizing agent that induces apoptosis secondary to G2/M arrest [62-63], doxorubicin is thought to mainly induce apoptosis through DNA intercalation with subsequent Topoisomerase II mediated double strand breaks [64], and cisplatin induces apoptosis secondary to the formation of DNA adducts [65]. Given the importance of PAI-1 expression as a diagnostic marker in breast cancer and its potential utility in determining

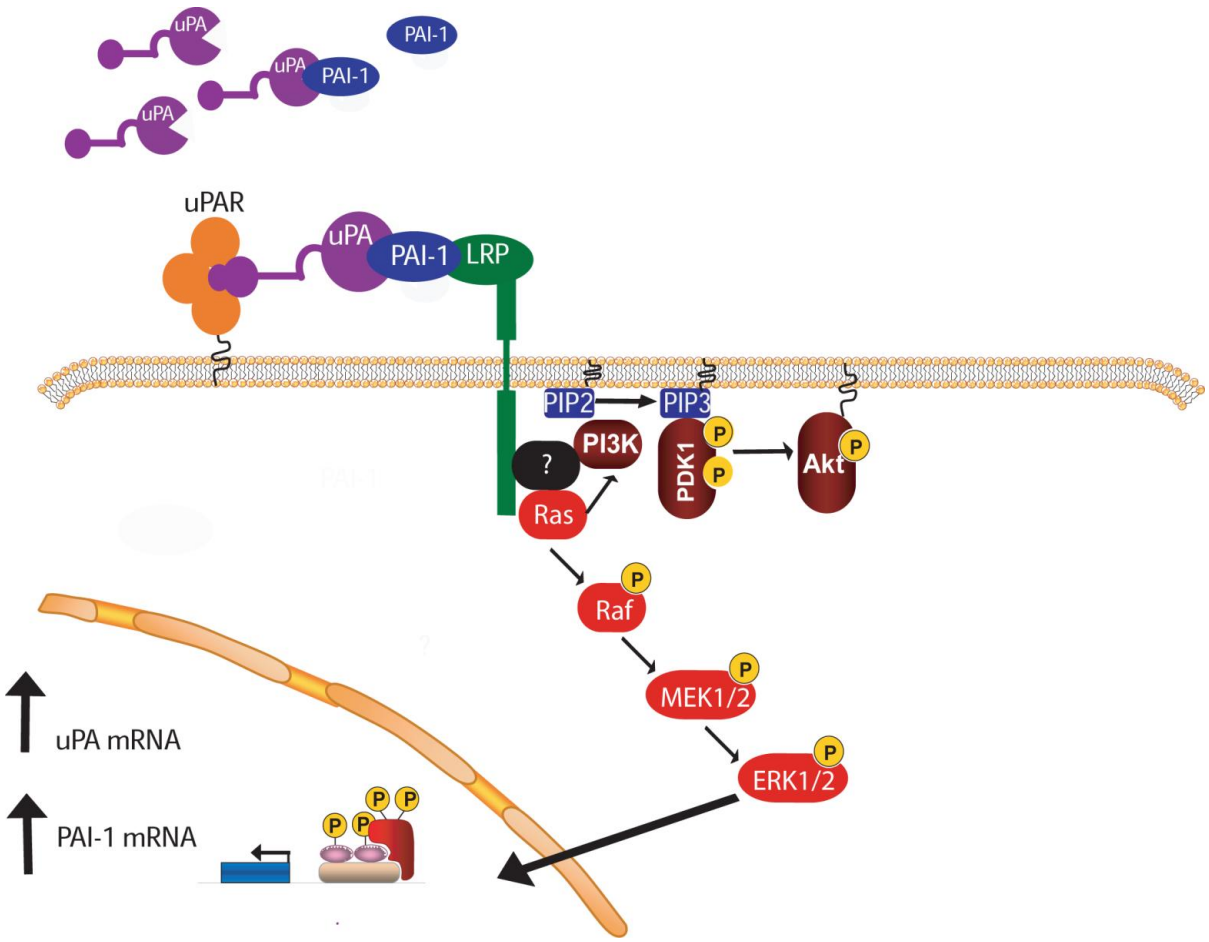

Figure 5.19. Working model of MAPK and PI3K signaling modification by PAI-1.
When uPAR/uPA/PAI-1 complexes are recognized on the cell surface by LRP, this triggers MAPK and PI3K signaling, with LRP functioning as the signaling module via an unknown intracellular co-factor. Activation of these pathways also positively regulates uPA and PAI-1 expression.

appropriate treatment strategies, it is also notable that all three of these agents are utilized in adjuvant chemotherapeutic regimens for breast cancer patients.

Taken together, our findings suggest that the observed elevation of uPA and PAI-1 antigen in breast cancer may represent more than a poor prognostic marker. Instead, our findings suggest the presence of both uPA and PAI-1 in the tumor microenvironment may directly protect breast cancer cells from apoptosis. Furthermore, we report for the first time that PAI-1, in a cell based model in which uPA is also present, protects breast cancer cells from spontaneous apoptosis in low serum and PAI-1 protects cells from the cytotoxic effects of three distinct chemotherapeutic agents: paclitaxel, doxorubicin, and cisplatin. Our findings further illustrate the importance of assessment of uPA and PAI-1 levels in breast cancer when determining treatment strategies and the need for continued research toward utilization of PAI-1 as a potential therapeutic target.

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Chapter VI

Discussion and Future Directions

Serine proteases and serine protease inhibitors (serpins) have long been recognized for their role in hemostasis and fibrinolysis. The balanced activities of thrombin, which activates fibrinogen to form fibrin clots, and APC, which inactivates other pro-coagulant factors, allows for tightly regulated deposition of fibrin clots at the site of a vessel injury. To resolve fibrin clots, the serine proteases uPA and tPA activate plasmin, which catalyzes degradation of fibrin. To prevent excessive fibrinolysis, uPA and tPA activity is balanced by the inhibitory capacity of PAI-1. Outside of the vasculature, thrombin, APC, uPA, and PAI-1 have proven to also participate in multiple processes involved in cancer growth and metastasis. In this dissertation, I examined the potential contributions of APC, uPA, and PAI-1 to breast cancer pathophysiology. I first focused on confirming the pro-angiogenic capacity of APC and determining the mechanism by which APC also promotes breast cancer cell invasion. Second, I examined the effects of uPA and its inhibitor PAI-1 and breast cancer cell viability.

While there have been previous reports on the ability of APC to promote angiogenesis, there is some debate over the physiological relevance of previous reports due to use of high concentrations of APC. In demonstrating that APC can promote HUVEC chemotaxis, invasion, and tube formation at low APC concentrations (1-10 μg/ml) we provide further evidence that APC is pro-angiogenic. Furthermore, studies with the *ex vivo* aortic ring sprouting assay confirm what others have suggested, that APC induced angiogenesis is dependent upon MMP activity and EGFR transactivation.

Although extensive work has been performed regarding the role of APC in angiogenesis, there are few previous reports on the capacity of APC to promote cancer cell motility. Originally, it was suggested that APC promotes motility of ovarian and

choriocarcinoma cancer cells by binding to PAI-1, thereby increasing uPA catalytic activity. In contrast, in breast cancer cells, I showed that APC promotes invasion independently of uPA and PAI-1 activity. The dependency of APC promoted cancer cell invasion on MMP activity and EGFR trans-activation parallels the mechanism demonstrated with endothelial cell motility by others. However, I found that while MMP activity is required for breast cancer cell invasion, APC does not significantly up-regulate MMP expression as it does in endothelial cells. Furthermore, I am first to report that APC activates MAPK and PI3K signaling in breast cancer cells, and that activation of these pathways is required for APC induced invasion.

Given the dearth of previous work on APC induced cancer cell migration, many questions remain as to the relative significance of APC to breast cancer pathology and the mechanism by which APC promotes cancer cell invasion. As reviewed in Chapter II, increased thrombin activity has been demonstrated in a wide variety of human cancers. In mouse models of cancer, thrombin activity has also been linked to metastasis. Despite the fact that thrombin activates APC, much less is known about APC activity in human cancers or how APC may influence metastasis. To start, it would be interesting to perform immunohistochemistry on breast tumor biopsies and matched normal tissue to determine if APC levels are elevated in tumor samples and if so, where APC is localized. Furthermore, it would be useful to expand my study to alternate human and mouse breast cancer cell lines. This would require characterization of these cell lines as to their expression of PAR-1, EPCR, and EGFR as these three receptors appear necessary for APC promoted invasion. In addition to invasion, I demonstrated that APC activates both MAPK and PI3K signaling. These pathways are key regulators of cell proliferation and sensitivity to apoptosis. It would

therefore be interesting to examine the effects of APC on proliferation of breast cancer cells and sensitivity to apoptosis.

Much remains to be studied about the precise mechanism whereby APC activates MAPK and PI3K signaling. First, my study would be improved by the use of shRNA to stably knockdown EPCR, PAR-1, and EGFR to more stringently examine the requirement of all three receptors for APC induced signaling. Furthermore, the generation of a mouse breast cancer cell line that expresses PAR-1 and EGFR with stable EPCR knockdown could be utilized in mouse models, such as mammary fat pad injection, to study primary tumor growth and metastasis.

This work and that of others support a mechanism in which EPCR localizes APC to the cell surface and APC cleaves PAR-1. It remains unclear how PAR-1 can trans-activate EGFR and how PI3K can be activated by APC. It is possible that PAR-1 cleavage by APC activates α or β/γ subunits which activate c-Src. c-Src can mediate phosphorylation of the cytoplasmic tail of EGFR. An alternative mechanism whereby PAR-1 can activate EGFR is via activation of the TNF-alpha converting enzyme (TACE; ADAM-17) that processes heparin bound-EGF (HB-EGF). HB-EGF would bind to the ligand-binding domain of EGFR, leading to the phosphorylation of the receptor and activation of Ras. Either mechanism could account for PI3K activation, as c-Src and Ras can activate PI3K.

In Chapter V, I examined the role of uPA and PAI-1 in breast cancer cell viability utilizing MDA-MB-231 and MDA-MB-436 breast cancer cells. The overall goal of this project was an attempt to understand the breast cancer "PAI-1 paradox." Multiple clinical studies have demonstrated that elevation of both uPA and PAI-1 in breast cancer is associated with poor prognosis. It is logical that elevated uPA would be a poor prognostic

indicator given the numerous capacities of uPA to promote angiogenesis, tumor cell invasion, as well as induce cancer cell proliferation (reviewed in Chapter II). However, it is unclear why elevated PAI-1, which would presumably function to counteract uPA, is a poor prognostic indicator. Others have focused on the potential pro-angiogenic functions of PAI-1 to explain the PAI-1 paradox. Taking a different approach, I hypothesized that PAI-1 would directly modulate signaling that promotes the overall viability of the breast cancer cells.

There have been few previous reports on the dual effects of uPA and PAI-1 on breast cancer cell physiology. A central limiting factor in previous research is the labile nature of PAI-1. If it does not react with a protease, PAI-1 undergoes spontaneous transition to a latent state. In vitro, active PAI-1 has a half-life of between 1 and 2 hours [reviewed in 1]. Furthermore, many breast cancer cells, as well as other cancer cell types, do not make PAI-1. Therefore, previous research has relied heavily on the use of purified PAI-1 that is added to cells. Given the latency transition of PAI-1 within 1 to 2 hours, this limits the scope of analysis of the role of PAI-1 on cancer cell physiology. While a mutated form of PAI-1 that does not undergo latency has been generated, its protease inhibitor activity is slightly altered from wild type PAI-1 [2]. To avoid these difficulties, we relied heavily on MDA-MB-231 breast cancer cells and also utilized MDA-MB-436 breast cancer cells as they generate their own uPA and PAI-1, as well as express uPAR, the uPA receptor.

There is extensive- yet sometimes conflicting - literature investigating the role of uPA and PAI-1 in invasion and adhesion to the ECM. Using active site inhibitors, I showed that inhibition of uPA activity decreases invasion through basement membrane extract whereas inhibition of PAI-1 activity promotes invasion. With regard to adhesion, using a stable knockdown approach, my data supports previous studies suggesting uPA promotes adhesion

to vitronectin by increasing the affinity of uPAR to vitronectin. Alternatively, there is some debate in the field as to whether PAI-1 promotes de-adhesion to vitronectin specifically, by competing for the same binding site as uPA, or promotes de-adhesion to multiple matrix proteins by disrupting uPAR/integrin complexes. Utilizing stable PAI-1 knockdown, my data indicates PAI-1 is generally de-adhesive, as PAI-1 knockdown resulted increased adhesion to collagen IV, fibronectin, and vitronectin.

Previous studies utilizing transient knockdown technique suggest uPA mediates proliferative signaling through activation of MAPK in breast cancer cells. Here, utilizing a stable knockdown approach, I show that uPA knockdown does decrease MAPK signaling as well as demonstrate that uPA knockdown inhibits cell growth in low serum conditions over a seven day period. Interestingly, in partial contrast with previous reports that uPA promotes anti-apoptotic signaling, I did not observe sensitization to chemotherapeutic agents. This discrepancy is likely a consequence of either different degrees of uPA knockdown or different experimental approaches. This result is also surprising, given the observed knockdown of PAI-1 in the uPA knockdown cells. However, the cells may have a threshold level of PAI-1 that remains protective. To further contribute to the existing literature, it would be interesting to generate stable knockdown of uPA in other breast cancer cell lines, and further characterize what signaling pathways are altered as well as perform flow cytometry to determine if stable uPA knockdown slows rates of DNA synthesis, as one might expect downstream of reduced MAPK signaling.

What previous studies focused on uPA have not addressed, is the role of uPA proteolytic activity on cancer cell viability. Using a small molecule uPA inhibitor, I show that in fact, inhibition of uPA activity decreases growth of cells in low serum conditions and

has inhibitory effects on both MAPK and PI3K signaling. Inhibition of uPA activity could have many downstream consequences that could account for this effect, notably activation of MMPs and plasmin which can activate growth factors (reviewed in II). However, I hypothesized that the effects of inhibition of uPA activity were a consequence of reduced binding of PAI-1 to uPA at the cell surface.

My initial experiments using a PAI-1 inhibitory antibody to examine changes in MAPK and PI3K signaling and cell growth in low serum supported my hypothesis. My hypothesis was further supported finding that stable knockdown of PAI-1 lead to decreased MAPK and PI3K signaling as well as decreased cell growth in low serum conditions. Interestingly, experiments in low serum conditions indicated that Ki67 expression did not decrease while active Caspase-3 staining increased. One might expect decreased MAPK signaling would decrease Ki67 expression. However, because Ki67 is a general marker of proliferation, further experiments should be pursued to determine if PAI-1 knockdown decreases proliferation. This could be achieved by examining BrdU incorporation and flow cytometry to examine DNA synthesis activity and cell cycle disribution. The increased activation of Caspase 3 strongly indicated that PAI-1 knockdown does sensitize breast cancer cells to apoptosis. Given increased activation of Caspase 3 in low serum conditions, I hypothesized that PAI-1 knockdown would sensitize cells to apoptosis inducing chemotherapeutic agents. This hypothesis was supported, as MDA-MB-231 cells with PAI-1 knockdown were significantly sensitized to paclitaxel, doxorubicin, and cisplatin. This finding represents the first demonstration to date that PAI-1 knockdown can sensitize cancer cells to chemotherapeutics that are used clinically to treat breast cancer.

Clearly, our findings need to be expanded to more breast cancer cell lines. My finding that PAI-1 inhibition reduced the growth of MDA-MB-436 breast cancer cells and sensitized them to low dose chemotherapeutics suggests my observations apply to a separate uPA and PAI-1 expressing breast cancer cell line. I am currently generating MDA-MB-436 cells with stable PAI-1 knockdown for future analysis. As an alternative approach, it would be interesting to take a reverse approach and stably express wild type PAI-1 in another breast cancer cell line which expresses uPAR and uPA and examine the consequent effects on viability.

Mechanistically, many questions still remain as to how PAI-1 could be protective against apoptosis in breast cancer cells. One study has suggested that PAI-1 prevents apoptosis intracellularly by inhibiting Caspase 3. My finding of differential Caspase 3 activation in PAI-1 knockdown cells, as well as the observed effects of an extracellular PAI-1 inhibitory strongly contradict this hypothesis. Others have predicted that PAI-1 should inhibit PI3K signaling as a function of dissociating or destroying uPA/uPAR/integrin complexes, thereby dissociating integrins from matrix ligands. Again, my demonstration that PAI-1 knockdown cells have instead decreased PI3K activity contradicts this hypothesis. The most likely mechanistic target for PAI-1 anti-apoptotic activity is the LRP-1 co-receptor responsible for clearing uPA/PAI-1 complexes at the cell surface. Recently, the LRP-1 co-receptor has been reported to protect fibroblast from apoptosis [3]. It will be intriguing to see if stable knockdown of LRP-1 could mimic the phenotype of PAI-1 knockdown cells. I have obtained LRP-1 shRNA constructs for this future project.

Determining the relevance of these findings to breast cancer pathology will require further investigation. While there are conflicting reports, it is generally understood that the

tumor cells are not the primary source of PAI-1 in the breast tumor microenvironment. It would therefore be interesting to establish a co-culture system in which breast cancer cells with PAI-1 knockdown are cultured with adipocytes or fibroblasts which can continuously synthesize PAI-1. In this system, one could analyze the effect of exogenous PAI-1 on breast cancer cells. Future mouse models would also go far to elucidate the overall contribution of PAI-1 to tumor biology. This would best be achieved by the generation of control mouse breast cancer tumor cell lines and cells with stable PAI-1 knockdown. These tumors could be injected into the mammary fat pad of syngeneic wild-type mice as well as PAI-1 knockout mice to investigate the contributions of tumor produced versus host produced PAI-1 on tumor growth and metastasis.

Therefore, in conclusion of this dissertation, I have demonstrated that the hemostatic serine protease APC promotes both angiogenesis and invasion of breast cancer cells by a mechanism dependent upon MMP activity, EGFR activation, and activation of PI3K and MAPK signaling. I also demonstrate that APC promotes cancer cell invasion independent of the fibrinolytic serine protease uPA and its inhibitor PAI-1, which was previously suggested. Next, utilizing inhibitory and stable knockdown strategies, I have shown that uPA induces proliferative signaling not only through binding of its receptor uPAR, as has been previously shown, but also through its catalytic capacity, which is required for PAI-1 binding at the cell surface. Lastly, I provided evidence that the serine protease inhibitor PAI-1 modulates both proliferative and apoptotic responses and is protective against apoptosis inducing chemotherapeutic agents.

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Appendix

J.C. Carter, R.A. Campbell, J.A. Gibbons, M.W. Gramling, A.S. Wolberg, F.C. Church. Enhanced cell-associated plasminogen activator pathway but not coagulation pathway activity contributes to motility in metastatic breast cancer cells, J Thromb Haemost (2010) 8 1323-32

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ORIGINAL ARTICLE

Enhanced cell-associated plasminogen activator pathway but not coagulation pathway activity contributes to motility in metastatic breast cancer cells

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Summary. *Background:* Activation of tumor cell-associated coagulation and plasminogen activator pathways occurs in malignant disease processes, including breast cancer, and may promote metastatic activity. *Objectives/Methods:* To compare the coagulation and plasminogen activator pathways of normal and metastatic cells, we examined two cell lines from the MCF-10 family of breast cells: near-normal immortalized MCF-10A cells, and metastatic MCF-10CA1 cells. *Results:* MCF-10CA1 cell motility was significantly increased as compared with that of MCF-10A cells. The two cell types supported similar rates of factor Xa generation, plasma thrombin generation, and fibrin formation. MCF-10A cells produced a stable fibrin network, whereas MCF-10CA1 cells lysed the surrounding fibrin network within 24 h of network formation. Importantly, fibrin located proximal to (within 10 μm) the MCF-10CA1 cell surface lysed substantially faster than fibrin located 100 μm from the surface. MCF-10CA1 cells supported significantly increased plasmin generation rates as compared with MCF-10A cells, providing a mechanism for the increased fibrinolytic activity of these cells towards the fibrin network. Metastatic MCF-10CA1 cells had increased expression (mRNA and protein) levels of urokinase plasminogen activator (u-PA) and decreased levels of plasminogen activator inhibitor-1 as compared with MCF-10A cells. Blocking u-PA activity with the active site-directed protease inhibitor amiloride substantially decreased MCF-10CA1 cell motility. Phosphorylated Akt levels were elevated in MCF-10CA1 cells, which partially explains the increased u-PA expression. *Conclusions:* These results suggest that the tumor-associated plasminogen activator pathway, not the coagulation pathway, is a key distinguishing feature between metastatic MCF10-CA1 cells and normal MCF-10A cells.

Keywords: breast cancer, cell motility, plasminogen activator inhibitor-1, thrombin, urokinase.

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Introduction

The frequent association of coagulopathies with cancer suggests a mechanistic role for abnormal coagulation and/or plasminogen activator (PA) (fibrinolytic) pathways in cancer-associated pathology [1]. Several studies have measured increased expression of procoagulant [tissue factor (TF)] or PA [urokinase PA (u-PA) or PA inhibitor-1 (PAI-1)] activity in tumor cells, and correlated these findings with enhanced tumor cell processes, including motility and metastasis [2–7]. However, the specific mechanisms promoting tumor cell metastasis have not been fully elucidated for most tumor types, and therapeutics targeting metastasis are only just being developed. It is not fully understood whether the coagulation pathway or PA pathway, alone or together in concert, promotes a metastatic phenotype, or which is the best therapeutic target. Thus, it is of paramount importance to further characterize the molecular pathways differentiating benign and metastatic tumor cells and to describe similarities and differences in coagulation and PA pathways.

Coagulation is initiated by the exposure of active TF to blood, and formation of a factor VIIa–TF complex leads to the generation of FXa [8–11]. The cascade of reactions triggered by this initiating event culminates in the production of thrombin, which has multiple roles in clot formation, wound healing, inflammation, and cancer biology. In coagulation, thrombin

activates platelets and catalyzes the proteolytic conversion of fibrinogen to fibrin, producing a web-like mesh that reinforces the primary platelet plug. During wound healing and inflammation, thrombin induces cellular proliferation and has potent chemotactic activity for leukocytes [12]. Importantly, although only poorly understood, increased TF expression is correlated with a poor prognosis in cancer patients [4,13]. It has been proposed that abnormal TF expression on tumor cells contributes to tumor metastatic potential.

The PA system has dual roles in cancer, related to fibrinolysis and tumor cell migration [14,15]. The PA system is composed of the u-PA and tissue-type PA (t-PA) , two serine protease inhibitors (serpin) (PAI-1 and a_2-antiplasmin), and the cell surface receptor for u-PA (u-PAR) [14,15]. In normal physiology, the PA system functions to regulate hemostasis through fibrinolysis [16]. In the pericellular environment, u-PA is the main source of plasminogen activation, which results in degradation of extracellular membrane proteins. PAI-1 inhibits both u-PA and t-PA to further inhibit plasminogen activation [5–7,14,15]. In breast cancer, elevated levels of u-PA and, paradoxically, PAI-1 are indicative of decreased patient survival [17,18].

Our goal was to characterize the coagulation and PA pathways in non-metastatic and metastatic tumor cells, and to determine their roles in tumor cell motility. The MCF-10 family of breast cell lines was derived from a woman with benign fibrocystic breast disease, which led to the near-normal immortalized MCF-10A cells [19,20]. After transformation with *T24-Ha-ras*, more aggressive cell lines were derived, leading to the fully metastatic MCF-10CA1 cell line. Use of this cell family enabled us to directly compare mechanisms differentiating non-metastatic and metastatic properties. As expected, MCF-10CA1 motility was enhanced as compared with that of MCF-10A cells. Interestingly, the coagulation pathway was virtually identical in these cells; however, MCF-10CA1 cells supported higher rates of fibrinolysis than MCF-10A cells. Additionally, blocking u-PA activity reduced MCF-10CA1 cell motility. These results suggest that the downregulation of the PA pathway, but not the coagulation pathway, would attenuate the aggressive phenotype of the MCF-10CA1 cells.

Materials and methods

Cell culture

MCF-10A and MCF-10CA1 cells were obtained from F. R. Miller (Wayne State University, Detroit, MI, USA) [19,20]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (GIBCO, Invitrogen, Carlsbad, CA, USA) containing 5% horse serum (HyClone, Logan, UT, USA), 1% antibiotic/antimycotic (GIBCO, Invitrogen), 20 μg mL^{-1} epidermal growth factor (EGF) (Invitrogen), 50 ng mL^{-1} hydrocortisone, 100 ng mL^{-1} cholera toxin (CalBiochem, San Diego, CA, USA), and 10 μg mL^{-1} insulin (GIBCO, Invitrogen), as described previously [19,20]. Cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C.

Motility assay

Cell motility was determined using a 48-well chemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA), as described previously [21]. Briefly, the lower chamber wells contained DMEM/F12 plus 1 mg mL^{-1} fatty acid-free bovine serum albumin (BSA) (Sigma, St Louis, MO, USA), with or without 5 ng mL^{-1} EGF (Invitrogen). Cells (1×10^5) were serum-starved and then plated in the upper wells in DMEM/F12 containing 1 mg mL^{-1} fatty-acid free BSA, above a collagen IV-coated (Sigma) 10-μm porated polycarbonate membrane (Neuro Probe). Chambers were incubated at 37 °C for 6 h in a humidified atmosphere. Cells were fixed and stained with a Diff-Quick Stain kit (Dade-Behring, Newark, DE, USA). Cells that migrated to the undersurface of the membrane were examined microscopically at × 200 magnification. Cells in four fields were counted; values represent the average number of cells per well ± standard deviation. All conditions were measured in triplicate.

Normal pooled plasma preparation

Blood was collected from 30 healthy individuals (53% female; 40% non-Caucasian) into sodium citrate (0.105 M, 3.2%, pH 6.5) and corn trypsin inhibitor (18.3 μg mL^{-1}; Haematologic Technologies, Essex Junction, VT, USA) [22], and plasma was prepared as described previously [23].

TF activity

TF activity was measured by incubating cells with FVIIa and FX (100 pM and 135 nM, final, respectively) in the presence of 5 mM $CaCl_2$, and measuring FXa activity by a chromogenic substrate method, as previously described [24]. The inhibitory antibody against TF, HTF-1, was a generous gift from S. Carson (University of Nebraska) [25].

Thrombin generation in plasma

Thrombin generation was measured by calibrated automated thrombography, using Z-Gly-Gly-Arg-AMC fluorogenic substrate (Diagnostica Stago, Parsippany, NJ, USA) on a Fluoroskan Ascent fluorometer (ThermoLabsystem, Helsinki, Finland), as previously described [23,26], except that 80 μL of plasma and 20 μL of HEPES-buffered saline (HBS)/BSA were added to washed cell monolayers to initiate reactions (final plasma concentration of 67.7%). Thrombin generation parameters were calculated by calibrating against α_2-macroglobulin/thrombin (Diagnostica Stago), using THROMBINOSCOPE software version 3.0.0.29 (Thrombinoscope BV, Maastricht, The Netherlands).

Clot formation and lysis assays

Clot formation was initiated by incubating cells seeded in a 96-well plate with recalcified (10 mM, final) plasma (final plasma

concentration of 86.7%). Clot formation was detected by turbidity at 405 nm in a SpectraMax 340PC plate reader [27].

Structural analysis by laser scanning confocal microscopy

Cells were seeded in a Lab-Tek II Chamber #1.5 coverglass (Nalge Nunc International, Naperville, IL, USA) at 33 000 cells per well, and grown to 80–95% confluence. The AlexaFluor-488 protein labeling kit was from Invitrogen. AlexaFluor-488-labeled fibrinogen containing ~ 8 mol of fluorophore per mole of fibrinogen was prepared as previously described [27]. Clots were formed as for clotting assays (as described above), with the addition of 10 μg of AlexaFluor-488-labeled fibrinogen per 150 μL of plasma (3.2% of the total fibrinogen concentration). Clots were imaged as a function of time, as described previously [27].

Quantitative polymerase chain reaction (PCR)

RNA was isolated from MCF-10A and MCF-10CA1 cell pellets, using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen), following the manufacturer's protocol. After an initial 5 min of denaturation at 95 °C, cDNA was amplified over 40 cycles (denaturation at 95 °C for 20 s, annealing at 55 °C for 1 min, and elongation at 68°C for 30 s), using SYBER Green (Applied Biosystems, Foster City, CA, USA) and an Eppendorf thermocycler. Primer sequences were as follows: u-PA forward, 5′-GGC AGC AAT - GAA CTT CAT CAA GTT CC-3′; u-PA reverse, 5′-TAT TTC ACA GTG CTG CCC TCC G-3′; PAI-1 forward, 5′-AAT CAG ACG GCA GCA CTG TC-3′; PAI-1 reverse, 5′-CTG AAC ATG TCG GTC ATT CC-3′; u-PAR forward, 5′-ACA GGA GCT GCC CTC GCG AC-3′; u-PAR reverse, 5′-GAG GGG GAT TTC AGG TTA GG-3′; β-actin forward, 5′-ATC ATG TTT GAG ACC TTC AA-3′; and β-actin reverse, 5′-CAT CTC TTG CTC GAA GTC CA-3′. Ct values for each experiment were averaged, and target expression was normalized for each cell line relative to β-actin as a loading control. After normalization of each cell line's u-PA, PAI-1 and u-PAR expression to β-actin, relative expression was determined using the $2^{-\Delta\Delta Ct}$ method by normalizing MCF-10A expression to 1 [28].

Enzyme-linked immunosorbent assay (ELISA)

Media conditioned by 24 h of incubation with confluent MCF-10A and MCF-10CA1 cells in six-well plates was diluted 1 : 5 in HBS (pH 7.4). Total u-PA and PAI-1 antigen levels were assessed with ELISA kits from R&D Systems (Minneapolis, MN, USA) and Molecular Innovations (Novi, MI, USA), respectively, according to the manufacturer's protocols and standards. Total u-PAR levels were determined by diluting cell lysates derived from 5×10^5 MCF-10A and MCF-10CA1 cells 1 : 10, and assaying them with an ELISA from R&D systems, according to the manufacturer's protocols. Briefly, u-PA, PAI-1 and u-PAR values were determined by comparing colorimetric signals from samples with standard curves generated with purified u-PA, PAI-1 and u-PAR provided by the manufacturer.

Immunoblot analysis

Cells were grown to confluence in a six-well plate, and then washed in ice-cold 20 mM sodium phosphate (pH 7.4)/150 mM NaCl [phosphate-buffered saline (PBS)] after 24 h of serum starvation. Cell lysates were collected in ice-cold buffer containing 50 mM Tris, 0.1% sodium dodecylsulfate (SDS), 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 5 mg mL^{-1} aprotinin, and 5 mg mL^{-1} leupeptin, and kept on ice. Lysates were centrifuged to remove insoluble plasma membrane. Conditioned medium from treated cells was collected and concentrated using a centrifugal filter device (Amicon Ultracel 30k; Millipore, Billerica, MA, USA). Protein concentration was determined using the BioRad Protein DC assay (BioRad, Hercules, CA, USA). Proteins were separated by SDS polyacrylamide gel electrophoresis in 10% polyacrylamide, and electrotransferred to a poly(vinylidene difluoride) membrane. PBS containing 0.1% Tween-20 was used in all steps of immunoblot analysis. Each step was preceded by three 9-min washes at room temperature. Non-specific binding was blocked by 5% non-fat dry milk for 30 min at room temperature. Membranes were incubated at 4 °C overnight with primary antibody diluted 1 : 1000 (unless otherwise noted) in 1% non-fat dry milk. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody diluted 1 : 5000 in 1% non-fat dry milk. The membranes were exposed to luminal substrate for 1 min, covered in plastic wrap, and exposed to X-ray film. The primary antibodies were goat anti-u-PAR (sc-9793; Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-ERK2 (Santa Cruz Biotech), rabbit anti-PAI-1 (1 : 2000 dilution) (ASHPAI; Molecular Innovations, Novi, MI, USA), and rabbit anti-u-PA (#389; American Diagnostica, Stamford, CT, USA).

Indirect cell surface-associated u-PA activity assay

MCF-10A and MCF-10CA1 cells (1×10^4) were plated in a 96-well plate, and plasminogen activation was determined as described previously [29–31]. Plasminogen (0.06 μg uL^{-1}) (Chromogenix, Milan, Italy) in 100 mM Tris and 0.5% Triton X-100 (pH 8.8) was added to cells and incubated at room temperature for 30 min. Following incubation, 5 μL of supernatant was removed and added to another 96-well plate containing 74.8 μL of buffer (100 mM Tris, pH 8.8, with 0.5% Triton X-100) and 5 μL of amiloride (6 mM stock solution) to inhibit any residual u-PA activity. Plasmin chromogenic substrate (S-2251; Chromogenix) was then added to the well (100 μL total volume) and hydrolyzed by plasmin generated by

plasminogen cleaved by u-PA on the cell surface. The plasmin concentration was determined from the rate of color development at 405 nm, by comparison with a standard curve of purified human plasmin (Chromogenix). To evaluate phosphatidylinositol-3-kinase (PI3K)–Akt in MCF-10CA1 cells, following 24 h of serum starvation, cells were treated with 10–20 μM LY294002 (Biomol-Enzo Life Sciences, Plymouth Meeting, PA, USA) or control [dimethylsulfoxide (DMSO)] for 24 h at 37 °C. Cell lysates were prepared as described above, and immunoblot analysis was performed as described above, using primary antibodies of rabbit anti-human phosphorylated Akt (Ser573, #9721; Cell Signaling Technology (Boston, MA, USA) and rabbit anti-human total Akt (#9722; Cell Signaling).

Results

Cell motility studies comparing the MCF-10A and MCF-10CA1 cell lines

With a modified Boyden chamber model, MCF-10CA1 cells were significantly more motile than MCF-10A cells, both at baseline with BSA as the chemoattractant, and when EGF was used as the chemoattractant (Fig. 1). Thus, as predicted by properties previously reported in the literature, the metastatic MCF-10CA1 cells were substantially more motile than the near-normal parental cell line, MCF-10A.

Thrombin generation, clot formation and clot lysis studies comparing the MCF-10A and MCF-10CA1 cell lines

As previous studies [2,3,32,33] have correlated metastatic potential with cellular procoagulant activity (TF activity and/or thrombin-generating potential), we first compared the abilities of MCF-10A and MCF-10CA1 cells to support FXa and thrombin generation and fibrin formation. As shown in the inset to the upper panel of Fig. 2, these cells did not differ in their ability to generate FXa in a TF-dependent mechanism; an anti-TF inhibitory antibody similarly reduced FXa generation on both cell types (Fig. 2A inset; Table 1). Both cell types supported robust thrombin generation in plasma, with identical thrombin generation parameters (lag time, peak, and endogenous thrombin potential) (Fig. 2A; Table 1). Consistent with these findings on procoagulant activity, clot formation (onset, maximum rate, and final clot turbidity) was equally supported by both MCF-10A and MCF-10CA1 cells (Fig. 2B; Table 1).

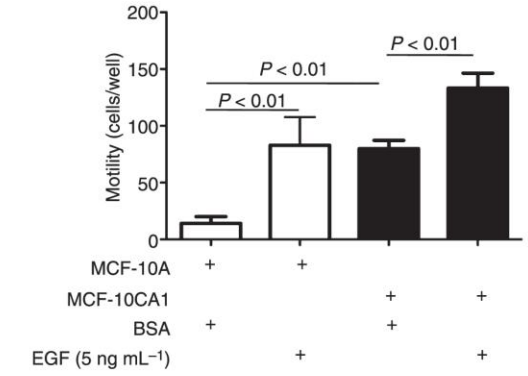

Fig. 1. MCF-10CA1 cells are more motile than MCF-10A cells. (A) Cells were serum-starved overnight, and then plated in the upper chamber of the modified Boyden chamber; random migration to bovine serum albumin (BSA) and chemotaxis to epidermal growth factor (EGF), respectively, were performed as described in Materials and methods. The data shown are the average of four separate experiments ± standard deviation.

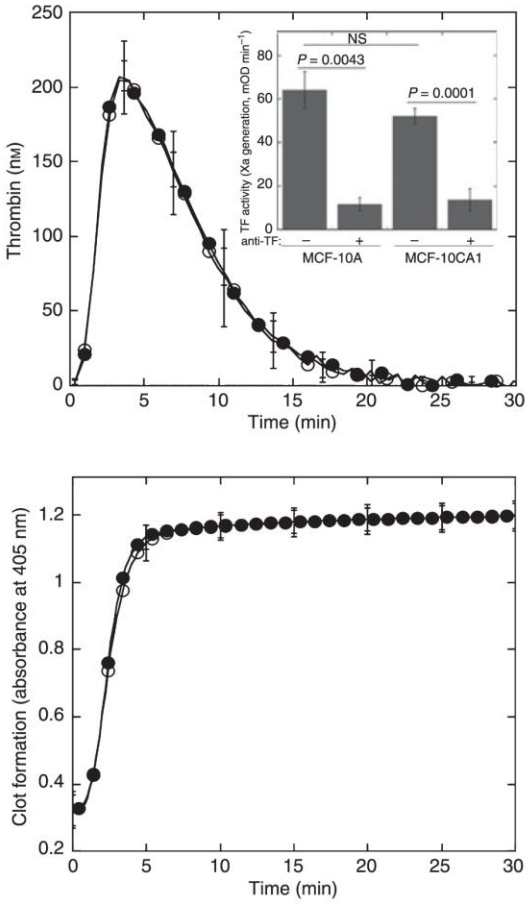

Fig. 2. MCF-10A and MCF-10CA1 cells support similar levels of tissue factor (TF) activity, thrombin generation, and fibrin formation. Recalcified (10 mM, final) plasma was added to confluent cell monolayers. Top panel: thrombin generation was measured using calibrated automated thrombography, as described in Materials and methods; the data [± standard deviation (SD)] shown are averaged from six separate experiments. Inset: TF activity was measured by incubating cells with FVIIa and FX in the presence of $CaCl_2$, as described in Materials and methods. Anti-TF inhibitory antibody (HTF-1) or non-immune control IgG was included as indicated. Bottom panel: fibrin polymerization was determined by turbidity at 405 nm; the data (± SD) shown are averaged from five separate experiments. Open circles: MCF-10A. Closed circles: MCF-10CA1. NS, not significant.

Table 1 Comparison of thrombin generation and fibrin clot formation in MCF-10A and MCF-10CA1 cells

Activity measured	MCF-10A	MCF-10CA1	*P*-value
TF activity (mOD min^{-1})*			
− Anti-TF antibody	64.1 ± 8.5	52.1 ± 3.6	0.09
+ Anti-TF antibody	11.6 ± 3.1	13.7 ± 5.3	0.58
P (+ anti-TF vs. − anti-TF)	< 0.0006	< 0.0005	
Thrombin generation			
Lag time (min)	1.1 ± 0.3	1.2 ± 0.2	0.58
Rate (nM min^{-1})	79.0 ± 14.7	83.4 ± 14.0	0.61
Time to peak (min)	3.8 ± 0.7	3.7 ± 0.4	0.78
Peak height (nM)	212 ± 22	210 ± 15	0.87
ETP (nM min)	1610 ± 240	1664 ± 118	0.74
Clot formation			
Clotting onset (min)	1.2 ± 0.3	1.2 ± 0.1	0.97
Fibrin polymerization rate (mOD min^{-1})	335 ± 41	355 ± 28	0.38
Final turbidity at 405 nm	0.89 ± 0.01	0.89 ± 0.02	0.91

mOD, milli-optical density; ETP, endogenous thrombin potential; TF, tissue factor. *Data show mean ± standard deviation for TF activity (n = 3), thrombin generation (n = 6), and clot formation (n = 5) assays.

We then directly examined the fibrin networks produced by these cells by allowing the cells to produce plasma-derived clots, and imaged the clots by laser scanning confocal microscopy 2 h after clotting was initiated. As expected from the turbidity data, both cell types produced dense fibrin networks, although the network produced near the MCF-10CA1 cell surface appeared to be slightly denser than that produced by MCF-10A cells (Fig. 3, upper panels). Together, these findings indicate that the differences between the motility of MCF-10A cells and that of MCF-10CA1 cells (Fig. 1) were not due to differences in cellular procoagulant activity.

Several previous studies have correlated metastatic potential with cellular PA activity [5,14,15,34,35]. Interestingly, when we imaged the clots produced by MCF-10A and MCF-10CA1 cells 24 h after initiation of clotting, we found that the fibrin network produced by MCF-10CA1 cells was fully lysed, whereas the fibrin network produced by the near-normal MCF-10A cells was only beginning to show signs of lysis (lighter, open regions in the network; Fig. 3). We confirmed the cellular origin of this fibrinolytic activity by examining fibrin network structure as a function of distance from the cell surface 4 h after clotting had been initiated. For both cell types, the fibrin network located 100 μm above the surface was intact (Fig. 4). The fibrin located near the surfaces of MCF-10A cells showed minor fibrinolytic degradation, whereas clots formed by the MCF-10CA1 cells showed complete lysis at the same distance from the cell surface (Fig. 4). These findings indicate that, as compared with MCF-10A cells, MCF-10CA1 cells

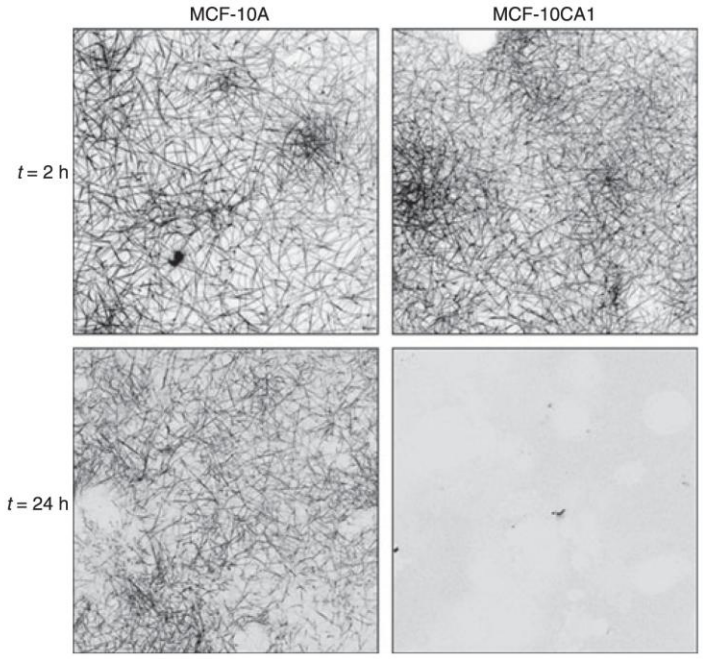

Fig. 3. MCF-10CA1 cells lyse the fibrin network within 24 h of clot initiation. Clots were formed as described in Materials and methods, by incubating recalcified plasma with confluent cell monolayers in the presence of AlexaFluor-488-conjugated fibrinogen to visualize fibrin via laser scanning confocal microscopy. Top panels: three-dimensional projections (146 × 146 μm, x–y) show fully formed fibrin architecture in 10-μm stacks just above the cell surface 2 h after clotting was initiated. Darker areas show increased fibrin density. Bottom panels: images in a single z-plane just above the cell surface show that MCF-10CA1 cells fully lysed the fibrin network 24 h after the initiation of clotting, whereas MCF-10A cells were only beginning to lyse the network (lighter patches). Each image is from one experiment, representative of three or four separate experiments.

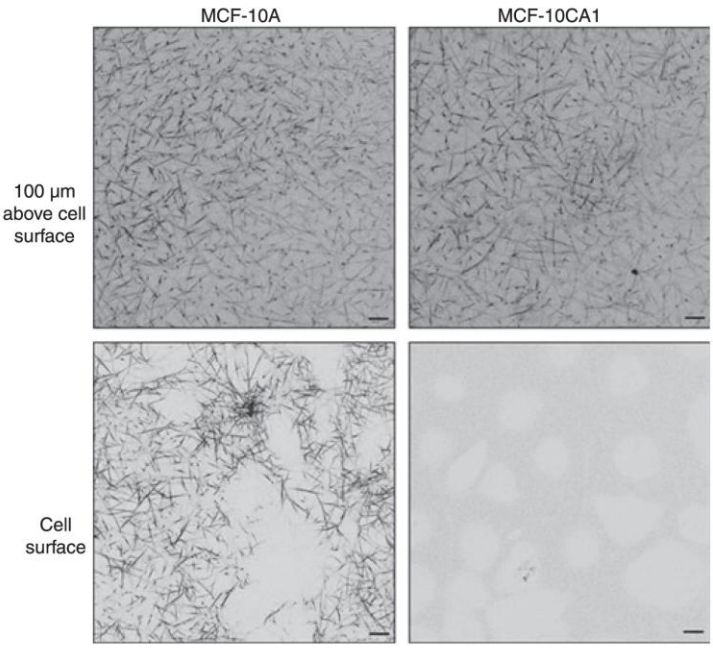

Fig. 4. Fibrinolytic activity originating from the MCA-10CA1 cell surface rapidly promotes lysis of the fibrin network. Clots were formed as described in Fig. 3. Clots were imaged in single *z*-planes at and 100 μm above the cell surface 4 h after clotting was initiated. Images from individual *z*-planes are shown, and indicate complete fibrinolysis of networks formed by MCF-10CA1 cells, but substantially less lysis of the network produced by MCF-10A cells.

exhibit high endogenous fibrinolytic activity, and that clots are lysed first in regions near the cell (the source of PA activity) and subsequently in more distal regions.

Expression of the PA system components in the MCF-10A and MCF-10CA1 cell lines

As the clot lysis studies indicated that a major difference between cell lines was the PA pathway, we specifically probed the PA mechanisms operant on these cells. Assessment of cell surface plasminogen activation indicated that MCF-10CA1 cells had significantly higher plasmin-generating activity than MCF-10A cells (Fig. 5, top left panel). There was significantly elevated mRNA expression of the PA factors u-PA and u-PAR in the MCF-10CA1 cells relative to MCF-10A cells, as determined by quantitative PCR. Furthermore, there was significantly decreased mRNA expression of the protease inhibitor PAI-1 in the MCF-10CA1 cells (Fig. 5, top right panel). Relative to MCF-10A cells, MCF-10CA1 cells expressed significantly more u-PA protein and significantly less PAI-1 protein in conditioned media as determined by ELISA (Fig. 5, bottom left panel). It is also of note that MCF-10CA1 cells exhibited slightly elevated expression of u-PAR protein as determined by ELISA of cell lysates (data not shown). Finally, immunoblot of conditioned media confirmed the presence of elevated u-PA and decreased PAI-1 expression in MCF-10CA1 cells relative to MCF-10A cells (Fig. 5, bottom right panel). These findings confirm that a major difference between the metastatic MCF-10CA1 cells and the near-normal MCF-10A cells is their increased PA activity, and identify this activity as a u-PA/PAI-1 imbalance that favors u-PA-mediated processes.

Altering u-PA activity and synthesis in MCF-10CA1 cells

We used the active site-directed inhibitor of u-PA, amiloride [36,37], to determine whether inhibition of u-PA activity decreases cell motility in MCF-10CA1 cells. Cell surface plasminogen activation on MCF-10CA1 cells was significantly reduced by amiloride (Fig. 6, top panel). Furthermore, in the presence of amiloride, MCF-10CA1 cell motility was significantly decreased, suggesting that u-PA activity is at least partly responsible for the increased chemotaxis in MCF-10CA1 cells (Fig. 6, middle panel). To begin to understand how MCF-10CA1 cells have increased u-PA levels as compared with MCF-10A cells, we studied the PI3K–Akt axis by measuring phosphorylated Akt levels (MCF-10A cells show virtually no expression of phosphorylated Akt; data not included). Previously, we showed that activation of PI3K–Akt results in increased u-PA levels and decreased PAI-1 levels [38]. When MCF-10CA1 cells were treated with LY294002 to inhibit PI3K–Akt, PA activity was substantially decreased (Fig. 6, bottom panel). Reduced expression of u-PA protein was correlated with reduced expression of phosphorylated Akt

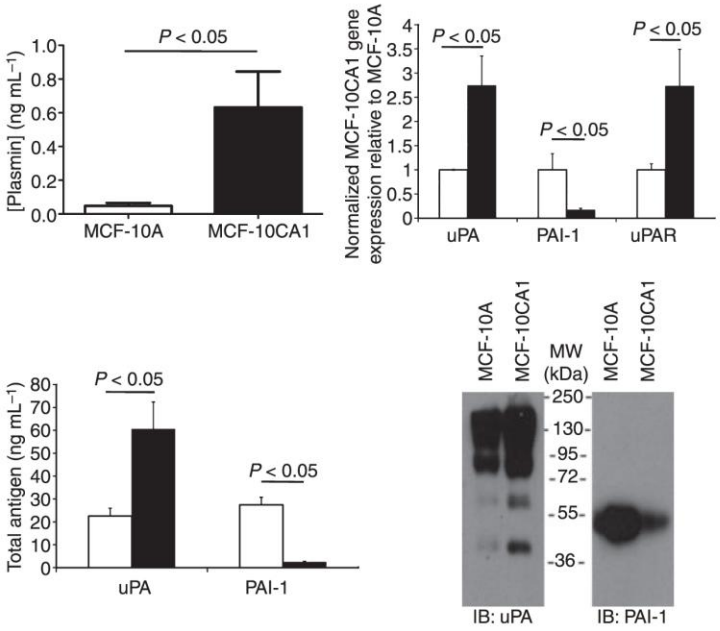

Fig. 5. Plasmin generation is significantly higher in MCF-10CA1 cells than in MCF-10A cells, and this is explained by an imbalance between urokinase plasminogen activator (u-PA) and plasminogen activator inhibitor-1 (PAI-1). Top left panel: plasminogen activation was measured as described in Materials and methods. Experiments were performed in triplicate; the data [± standard deviation (SD)] shown are averaged from four separate experiments. Top right panel: quantitative polymerase chain reaction analysis of u-PA, PAI-1 and u-PA receptor (u-PAR) mRNA in the MCF-10A cells (white bars) and MCF-10CA1 cells (black bars). Primer sequences, reaction conditions and determination of relative expression with the $2^{-\Delta\Delta Ct}$ method were as described in Materials and methods, and the data (± SD) shown are averaged from three separate experiments. Bottom left panel: enzyme-linked immunosorbent assays for total u-PA and PAI-1 antigen in conditioned media derived from the MCF-10A cell line (white bars) and MCF-10CA1 cell line (black bars) were performed as described in Materials and methods. Experiments were performed in duplicate; the data (± SD) represent the average of three separate experiments. Bottom right panel: representative immunoblots of u-PA and PAI-1 in conditioned media derived from MCF-10A and MCF-10CA1 cells. Probing for u-PA reveals, as expected, multiple species, including low molecular mass u-PA, active and higher molecular mass u-PA species, and the covalent u-PA–PAI-1 complex.

following LY294002 treatment (Fig. 6, inset in bottom panel). Treatment with LY294002 also significantly reduced MCF-10CA1 wound-induced migration as compared with the DMSO vehicle control treatment (data not shown). Although the motogenic capacity of the MCF-10CA1 cancer cells was not completely inhibited in these experiments, these results show that inhibition of u-PA activity or expression significantly reduces MCF-10CA1 cell motility.

Discussion

Changes in the coagulation and PA pathways are frequently detected in cancer. Alterations in the coagulation pathway typically result from the upregulation of TF on the tumor cell surface to enhance thrombin generation [2,3,32,33], whereas alterations in the PA pathway typically result from upregulation of u-PA or PAI-1, or even u-PAR [14,15]. The signaling pathways triggered by increased thrombin or u-PA/PAI-1 expression drastically alter the tumor cell microenvironment to promote tumor cell processes, including invasion and metastasis. Heterogeneity in the molecular composition of tumors from different cellular sources complicates studies aimed at isolating and identifying specific mechanisms promoting tumor metastasis. Although MCF-10A cells are widely viewed as near-normal breast epithelial cells, and are often compared with malignant epithelial cell lines [39–42], few studies have compared MCF-10A cells with the metastatic MCF-10CA1 cells derived from the MCF-10A cells. Thus, the well-characterized origin of MCF-10A and MCF-10CA1 cells makes these cells ideal for studying the specific contributions of the coagulation and PA pathways that differentiate non-metastatic and metastatic breast epithelioid cells.

In the present study, we found similar TF and total procoagulant activities in the normal MCF-10A and metastatic MCF-10CA1 cells. Indeed, the level of TF activity measured on both of these cell types was typical of extravascular cells (e.g. fibroblasts) [43]. These findings contrast with several prior studies [2,3,32,33], which noted increased TF activity in metastatic cells as compared with non-metastatic cells. Given the profound differences in cell motility (Fig. 1) and metastatic phenotype [19,20] between MCF-10A and MCF-10CA1 cells, this finding shows that the mechanism(s) promoting tumor metastasis in these cells does not necessarily involve or require increased TF or thrombin generation.

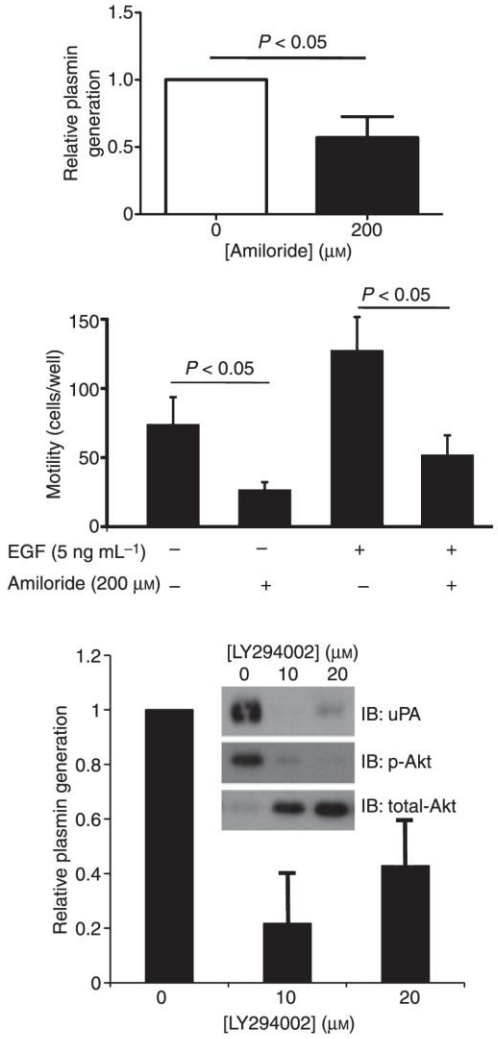

Fig. 6. Altering urokinase plasminogen activator (u-PA) activity (amiloride) and synthesis (LY294002) in MCF-10CA1 cells. Top panel: plasminogen activator activity with amiloride and normalized to control as described in Materials and methods. The data [± standard deviation (SD)] shown are averaged from three separate experiments performed in triplicate. Middle panel: cell motility with amiloride or dimethylsulfoxide control was measured as detailed in Materials and methods. The data (± SD) shown are averaged number of cells per well from three separate experiments. Bottom panel: LY294002 treatment reduces plasminogen activator activity and phosphorylated Akt (p-Akt) expression in MCF-10CA1 cells. The data (± SD) shown are averaged from three separate experiments performed in triplicate and normalized to no-treatment control as described in Materials and methods. Inset: Immunoblot (IB) for u-PA expression from conditioned medium, and phosphorylated Akt and total Akt protein from cell lysates following LY294002 treatment. EGF, epidermal growth factor.

In contrast to the similarities in procoagulant activity between MCF-10A and MCF-10CA1 cells, our findings showed significant upregulation of the PA pathway in metastatic MCF-10CA1 cells as compared with non-metastatic MCF-10A cells. Specifically, MCF-10CA1 cells had higher u-PA protein levels but lower PAI-1 protein levels than MCF-10A cells. This alteration in the u-PA/PAI-1 ratio enabled MCF-10CA1 cells to support significantly higher levels of plasmin generation (Fig. 5) than MCF-10A cells. The functional consequences of this increased PA phenotype in the metastatic cells was readily apparent in their ability to rapidly dissolve a fibrin network (Figs 3 and 4). Comparing MCF-10CA1 cells with MCF-10A cells, our results are consistent with the hypothesis that alteration of the u-PA/PAI-1 ratio to favor u-PA expression and increased PA activity promotes increased tumor invasiveness.

Direct support for this hypothesis comes from our experiments in which u-PA activity and expression on MCF-10CA1 cells were inhibited. u-PA has long been known to be a key mediator in tumor cell biology. u-PA participates in cytoskeletal rearrangement in tumor cells [44] and induces epidermal tumor cell proliferation [45]. u-PA bound to u-PAR promotes migration and invasion of a number of cancer cell types. By blocking u-PA activity with the u-PA inhibitor amiloride [36], we significantly reduced MCF-10CA1 cell motility. These results show that increased u-PA activity is positively correlated with motility in MCF-10CA1 cells *in vitro*, and imply that inhibition of u-PA tumor cell-associated activity could reduce the motility of cells *in vivo*.

Whereas the u-PA–u-PAR complex is a cell motogenic factor owing to plasminogen activation, u-PA and u-PAR are also involved in cell adhesion, which influences motility. u-PAR binds the extracellular matrix protein vitronectin [46], and this affinity is increased in the presence of u-PA [47]. Therefore, interference with the binding of u-PAR to vitronectin can alter adhesion and subsequently motility. Edwards *et al.* [48] found that inhibition of u-PA activity with amiloride reduced adhesion of lymphocytes to vitronectin. Furthermore, u-PA can cleave u-PAR [49], and amiloride could alter adhesion to vitronectin. However, in our experiments, motility was assessed in the presence of purified collagen. Although it is possible that u-PA–u-PAR binding to vitronectin produced by the MCF-10CA1 cells themselves may contribute to our motility observations, we feel that the decreased motility observed in the presence of amiloride is primarily a function of decreased u-PA proteolytic activity.

MCF-10A cells were transformed with oncogenic Ras, an upstream activator of PI3K [50]. As the PI3K–Akt signaling pathway has been implicated in u-PA and PAI-1 expression, and tumor cell survival, proliferation, and migration [50,51], we treated MCF-10CA1 cells with the PI3K signaling inhibitor LY294002 to investigate the increased u-PA expression in these cells. Our results showing increased Akt phosphorylation and u-PA expression in MCF-10CA1 cells are consistent with those of Shukla *et al.* [52], who showed that PI3K–Akt pathway activation was linked to upregulation of u-PA expression in prostate cancer cells, and Whitley *et al.* [38], who reported a positive effect of u-PA expression due to PI3K–Akt pathway expression in SKOV-3 ovarian cancer cells. Together, these

findings implicate a common mechanism by which increased PI3K–Akt activity increases u-PA expression and activity, promoting an aggressive phenotype, as seen by increased cell motility in the MCF-10CA1 cells.

In summary, understanding the relationship between the coagulation and/or PA pathways in cancer progression is of vital importance. Furthermore, it is important to describe coagulation and PA pathways in tumor cell lines of different lineages and metastatic potential. There is considerable debate about the overall effect of thrombosis and its link to the progression of cancer; this is centered on the type of cancer being studied, the anticoagulant(s) being used, and the treatment regimens for the patient population, regarding both chemotherapy and anticoagulation (human or animal models) [53,54]. Both the coagulation pathway and the PA pathway have been targeted for adjunctive chemotherapy; novel u-PA small molecule inhibitors are currently in clinical trials for the treatment of different cancers, including breast cancer [55,56]. Our results obtained by comparing the normal MCF-10A cells with the related and more aggressive MCF-10CA1 cells show that we still have much to learn regarding the interplay between coagulation and PA pathways in breast and other cancer types.

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Disclosure of Conflict of Interests

A.S. Wolberg has received honoraria and grant support from Novo Nordisk A/S in the last 12 months. The other authors state that they have no conflict of interest.

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