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Breast cancer and metabolic syndrome linked through the plasminogen activator inhibitor-1 cycle

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Summary

Plasminogen activator inhibitor-1 (PAI-1) is a physiological inhibitor of urokinase (uPA), a serine protease known to promote cell migration and invasion. Intuitively, increased levels of PAI-1 should be beneficial in down-regulating uPA activity, particularly in cancer. By contrast, in vivo, increased levels of PAI-1 are associated with a poor prognosis in breast cancer. This phenomenon is termed the “PAI-1 paradox”. Many factors are responsible for the upregulation of PAI-1 in the tumor micro-environment. We hypothesize that there is a breast cancer predisposition to a more aggressive stage when PAI-1 is upregulated as a consequence of Metabolic Syndrome (MetS). MetS exerts a detrimental effect on the breast tumor microenvironment that supports cancer invasion. People with MetS have an increased risk of coronary heart disease, stroke, peripheral vascular disease and hyper-insulinemia. Recently, MetS has also been identified as a risk factor for breast cancer. We hypothesize the existence of the “PAI-1 cycle”. Sustained by MetS, adipocytokines alter PAI-1 expression to promote angiogenesis, tumor-cell migration and procoagulant micro-particle formation from endothelial cells, which generates thrombin and further propagates PAI-1 synthesis. All of these factors culminate in a chemotherapy-resistant breast tumor microenvironment. The PAI-1 cycle may partly explain the PAI-1 paradox. In this hypothesis paper, we will discuss further how MetS upregulates PAI-1 and how an increased level of PAI-1 can be linked to a poor prognosis.

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Introduction

Western diet, Metabolic Syndrome (MetS) and breast cancer

“When diet is wrong medicine is of no use. When diet is correct medicine is of no need.” Ancient Ayurvedic Proverb

Breast cancer is the second leading cause of cancer deaths among women in the United States. It is predicted that there will be more than 178,000 new cases of invasive breast cancer and more than 40,000 estimated deaths from breast cancer in 2007.⁽¹⁾ The lifetime probability of a woman developing breast cancer is 1 in 7.⁽²⁾ The duration of exposure to estrogen is a major risk factor for breast cancer.⁽²⁾ Modifiable risk factors for breast cancer include obesity, excessive alcohol use and physical inactivity.⁽²⁾ Invasion and metastasis are significant hindrances to the effective treatment of women with breast cancer.^(3,4) Despite advances in detection and therapy, many women will return with distant metastases and eventually succumb to their disease. Understanding the mechanisms that promote or permit invasion is crucial to the development of effective therapies.

Obesity and the resulting associated metabolic pathologies, now termed Metabolic Syndrome (MetS), affect more than 50% of adults in the United States. MetS is a group of metabolic risk factors, including central obesity, dyslipidemia, hyperinsulinemia, hypertension and prothrombotic/proinflammatory states.^(5,6) MetS increases the risk of coronary heart disease, stroke, peripheral vascular disease and hyperinsulinemia.^(7,8) There is growing epidemiological data to suggest a correlation between high-fat consumption from a Western diet and breast cancer in humans.^(9–11) Recently, it was shown that obesity and weight gain are associated with increased breast cancer recurrence and cancer mortality.^(12–14)

In the breast, adipocytes are responsible for the synthesis of adipocytokines, potent cellular modulators upregulated during MetS.^(7,8,15–19) Besides adipocytes, there are vascular endothelial cells and stromal fibroblasts that can be regulated by adipocytokines.⁽²⁰⁾ In addition, a prominent feature of MetS is the upregulation of the serine protease inhibitor (serpin), plasminogen activator inhibitor-1 (PAI-1; systematic name, SERPINE1).^(20–23) PAI-1 is a physiological inhibitor of urokinase-type plasminogen activator (uPA), a serine pro-tease involved in the promotion of cellular de-adhesion, migration/invasion and activation of plasmin from plasminogen.^(24–27) uPA is upregulated in breast cancer as it progresses to a metastatic disease. Increased levels of the protease are associated with a poor prognosis.⁽²⁸⁾

Instinctively, one would predict that increased expression of PAI-1 might be beneficial in breast cancer, since it potently inhibits uPA proteolytic activity. In vitro, PAI-1 has been shown to inhibit the effects of uPA in cancer.^(24–27) However, several clinical studies have found that increased PAI-1 expression in breast cancer is associated with a grim prognosis.⁽³⁾ In the following sections, we present a hypothesis to possibly explain the relationship of MetS to breast cancer, via a link with PAI-1. We hypothesize that MetS and adipocytokines alter the microenvironment surrounding the tumor cell by changing the

expression of various factors, including PAI-1, that promote breast cancer cell migration and invasion (Figs 1 and 2).

Plasminogen activator system and breast cancer

The plasminogen activator (PA) system plays an important role in promoting tumor cell invasion.^(24–27) uPA is a serine protease involved in many cellular processes including motility, activation of plasminogen to plasmin and activating pro-matrix metalloproteases (MMPs). While bound to the urokinase receptor (uPAR), uPA activates the zymogen plasminogen to the serine protease plasmin at the cell surface. Plasmin activates MMPs and degrades the surrounding extracellular matrix (ECM).^(24–27) Serpins control proteolytic activity during normal and pathological processes.^(29,30) There are four serpins that regulate the serine proteases of the PA system, PAI-1, plasminogen activator inhibitor-2, plasminogen activator inhibitor-3 (also known as protein C inhibitor) and α -plasmin inhibitor. An imbalance in the ratio of protease to the inhibitor allows for increased degradation of the ECM and increased invasion of tumor cells, which explains why many components of the PA system are associated with poor prognosis in several types of cancer.^(24–27)

There is an inherent balance among uPA, uPAR and PAI-1 to regulate focal ECM proteolysis and invasion.⁽³¹⁾ However, the known biochemical function of PAI-1 contradicts its pathological function in cancer. An increased level of PAI-1 would be expected to inhibit uPA generation of plasmin, protecting the ECM from proteolysis and thus inhibiting invasion. By contrast, high PAI-1 levels correlate with a poor prognosis and reduced survival in many cancers including breast cancer.⁽³⁾ Analyses of pooled data from 18 separate studies showed unequivocally that high levels of PAI-1 and uPA in the tumor are associated with an increase in relapse and metastasis, and a decrease in survival, indicative of a poor prognosis in primary breast cancer.⁽³⁾ Furthermore, a prospective randomized investigation found that the levels of PAI-1 and uPA in primary breast cancer are predictive of disease recurrence.⁽²⁸⁾

PAI-1 is expressed in many cell types including endothelial cells, fibroblasts, adipocytes, smooth muscle cells, numerous tissue epithelial cells, keratinocytes, granulosa cells and platelets. PAI-1 is the primary inhibitor of the PA system targeting both uPA and tissue-type PA (tPA). PAI-1, in the presence of either VN or heparin, also inhibits the serine protease thrombin, although the importance of this inhibition reaction in cancer biology is not well understood. PAI-1 is found in trace levels in peripheral blood because it is a potent uPA and tPA inhibitor and its latent and protease-complexed forms are rapidly cleared. PAI-1 is conformationally unstable, rapidly decaying to an inactive form within 1 to 2 hours.⁽³²⁾ Binding to VN will stabilize PAI-1 for 4 to 6 hours.^(33,34) PAI-1 will rapidly inhibit uPA, which is bound to urokinase receptor (uPAR) on the cell surface. The PAI-1:uPA:uPAR ternary complex interacts with the low density lipoprotein receptor-related protein (LRP), which internalizes the quaternary complex.⁽³⁵⁾ uPAR and LRP will recycle to the cell surface while PAI-1 and uPA are degraded.⁽³⁶⁾

The “PAI-1 paradox” exists because increased levels of PAI-1, which presumably would downregulate uPA activity, should be considered good for cancer. However, in vivo, PAI-1 is associated with a poor prognosis in breast cancer. An increase in PAI-1 levels in the breast tumor environment will affect cell adhesion, migration and invasion, apoptosis and proliferation, cell signaling and tumor angiogenesis via a mechanism that is partly independent of its inhibitory site.^(24–27,37) We suggest the concept that increased levels of PAI-1 involve both “traditional” and “non-traditional” roles for PAI-1 in cancer biology.

The hypothesis: PAI-1 cycle

PAI-1 cycle links MetS with breast cancer

We propose that PAI-1 is upregulated as a consequence of MetS. This increase in PAI-1 exerts a detrimental effect on breast tumor cells, adipocytes, stromal fibroblasts and vascular endothelial cells to promote tumor cell invasion. The “PAI-1 cycle” is sustained by MetS, resulting in the increased expression of PAI-1 in the tumor microenvironment (Fig. 1). This PAI-1 will advance numerous pathological processes of breast cancer (Figure 2). However, we do not presume that all people with MetS will be predisposed to breast cancer. Adipocytokines, whose production is promoted by MetS, will alter PAI-1 expression.^(7,8,18,38–40) The induction of insulin-resistance in adipocytes by MetS will also sustain PAI-1 expression.^(41,42) Increased levels of PAI-1 will support tumor angiogenesis,^(43–45) promote tumor cell adhesion/de-adhesion and activate signaling pathways to promote tumor cell invasion.^(24–27,46,47) PAI-1 will also promote the generation of pro-coagulant microparticles from endothelial cells. These microparticles can support the activation of prothrombin to thrombin.^(48–50) Thrombin facilitates tumor cell migration by activating protease-activated receptors to further promote PAI-1 synthesis.^(51,52) Additionally, fibrin produced near the tumor by thrombin will coat the tumor cells, disguising the tumor from the immune system.^(53,54) PAI-1 will also counter act apoptosis,^(55,56) which could increase breast cancer cell survival when treated with a chemotherapeutic drug. Ultimately, the PAI-1 cycle would provide a chemotherapy-resistant breast tumor microenvironment primed for invasion/metastasis^(55–59) (Figs 1,2). We will further discuss the various pathophysiological aspects of the PAI-1 cycle and its role in cancer.

Tumor angiogenesis

PAI-1 has been shown to be both pro-angiogenic and anti-angiogenic. PAI-1^{−/−} mice, described by Devy et al⁽⁶⁰⁾ and McMahon et al,⁽⁶¹⁾ have a reduction in angiogenesis, up to 60%,⁽⁶¹⁾ compared to wild-type mice. Addition of wild-type (wt)-PAI-1 at physiological concentrations, up to 1 nM, restores/increases angiogenesis up to 3-fold, as seen in two different angiogenic assays,^(60,61) while wt-PAI-1 above 1 to 2 nM reduces angiogenesis in these same assays.^(60,61)

How PAI-1 affects angiogenesis is not fully understood. Adenoviral gene transfer of wild-type and mutant forms of PAI-1 that either bind ineffectively to vitronectin (VN) or are unable to inhibit the PA system show angiogenesis is promoted through PAI-1 inhibition of uPA and tPA.^(60,62) uPA and tPA activate plasminogen to plasmin. Excessive plasmin

activity could lead to vessel destabilization.⁽⁶²⁾ Others have shown that the interaction of PAI-1 with the surrounding ECM is a key parameter affecting the progression of angiogenesis. In neuroblastoma tumors, PAI-1 co-expresses with integrins, specifically $\alpha_v\beta_3$, on endothelial cells.⁽⁶³⁾ PAI-1 binds to VN and prevents VN-integrin cell interactions, resulting in endothelial cell migration towards fibronectin.⁽⁶³⁾

Cell adhesion and proliferation

The PA system is involved in both promoting and preventing cell adhesion. Cells can bind VN through integrins, such as $\alpha_v\beta_3$ and uPAR.^(64,65) uPAR on the cell surface binds directly to VN^(65,66) and this interaction will upregulate integrin binding to the ECM. uPA bound to uPAR also promotes the interaction of uPAR to VN and strengthens cell adhesion to the ECM.^(67,68) Occupied uPAR will also associate with integrins to further strengthen the cell's adhesion to VN [reviewed in Refs 69,70].

PAI-1 inhibits PA-system-mediated cell adhesion by disrupting the interactions of uPAR and integrins with the ECM.^(64,65,71) PAI-1 competes with uPAR for VN binding.⁽⁶⁵⁾ PAI-1 will lose the ability to bind to VN when it complexes with uPAR-bound uPA.^(65,72) PAI-1 inhibition of uPA prevents the serpin from interacting with the cell and ECM, promoting cell adhesion to VN, fibronectin and laminin.⁽⁷³⁾ By removing PAI-1 from VN, cell adhesion is promoted through integrin interaction with the ECM. PAI-1 inhibition of uPA can also promote cell de-adhesion. Internalization of the integrin:uPAR:uPA:PAI-1 complex by LRP removes key components needed to adhere the cell to the ECM.⁽⁷⁴⁾

We have found that overexpressing wild-type PAI-1 (wt-PAI-1) in the MDA-MB-435 cells, a breast cancer cell line, decreased the rate of proliferation when compared to either parental cells or the inactive mutant P14 PAI-1-expressing MDA-MB-435 cells (P14 is T333R PAI-1).^(73,75,76) Anchorage-dependent colony-forming efficiency was used as a measure of cell proliferation. The wt-PAI-1-expressing MDA-MB-435 cells had a decreased ability to form colonies compared to the parental, Neomycin vector control and P14-PAI-1-expressing MDA-MB-435 cells (Table 1). Anchorage-independent proliferation, a hallmark of metastatic potential, had similar results. wt-PAI-1-expressing MDA-MB-435 cells had a reduced ability to form colonies in soft agar when compared with the other MDA-MB-435 cells (Table 1). Although PAI-1 has been associated with a negative prognosis in breast cancer, these results show it to be a negative regulator of proliferation of breast cancer cells in vitro.

A possible explanation for the decrease in proliferation by PAI-1 may be through an established function of the cell signaling protein, Akt. Akt is involved in cell survival and proliferation [reviewed in Ref. 77]. PAI-1^{-/-} endothelial cells have an increased level of phosphorylated Akt and an increased rate of proliferation.⁽⁷⁸⁾ wt-PAI-1-expressing MDAMB-435 cells have less phosphorylated Akt compared to inactive P14-PAI-1-expressing MDA-MB-435 cells, parental MDA-MB-435 cells and Neomycin vector control MDA-MB-435 cells (data not included). Our laboratory and others have recently shown that PAI-1 levels are increased in cells that have had the phosphatidylinositol 3-kinase/Akt-Akt signaling axis inhibited.^(42,79) Why a negative indicator of breast cancer survival slows

down cell proliferation is not fully understood. It is possible that PAI-1 may serve in a protective role; thus, slowing tumor cell growth could permit more genetic alterations to occur advancing breast cancer to a metastatic stage.

Cell migration and invasion

All components of the PA system are involved in regulating cell migration and invasion. These interactions of the PA system can be complex, cell-type-specific and often context specific. uPA stimulates migration of breast cancer cells, dependent on uPAR ligation and subsequent activation of cell signaling pathways by integrins.^(80,81) There is a reduction in tumor cell migration and metastasis when uPA is inhibited by PAI-1. When uPA is overexpressed, altering the ratio of protease to inhibitor, cell migration in vitro is promoted.⁽⁸²⁾

PAI-1 has been shown to promote cell migration. Chazaud et al⁽⁸³⁾ showed PAI-1 attaches to cells through uPA:uPAR, which alters cell morphology and promotes breast cancer cell migration. This increase in migration can only occur when all of the components of the PA system are present.⁽⁸³⁾ wt-PAI-1-expressing MDA-MB-435 cells are shown to have significantly increased chemotaxis to VN and fibronectin compared to control cells.⁽⁷³⁾ Alternatively, PAI-1 can support cell migration by preventing cell adhesion to VN.^(67,84) In smooth muscle cells, PAI-1 increases migration by binding to LRP, which results in morphology changes, cytoskeleton reorganization and alterations in signaling pathways.⁽⁸⁵⁾

PAI-1 also inhibits tumor cell migration.^(72,75,86–88) HT1080 cells expressing wt-PAI-1 have reduced cell migration in vitro and do not form many lung metastases in vivo.⁽⁸²⁾ The same is true in glioma cells expressing PAI-1 with a reduction in invasion in vitro compared to wild type.⁽⁸⁴⁾ Overexpressing PAI-1 in breast and ovarian cancer cells reduces both cell migration and invasion, dependent on the active form of PAI-1.⁽⁷⁵⁾ PAI-1 will complex with uPA and through the formation of a ternary complex with uPAR, will also bind LRP. This quaternary complex is internalized resulting in a loss of components of the PA system and reduction in cell migration.⁽⁸⁸⁾ Treatment with either receptor-associated protein (RAP), a LRP-binding protein that competitively inhibits ligand binding, or LRP-blocking antibodies reverses these effects on migration. However, PAI-1-mediated inhibition of migration can also be independent of its uPA-inhibitory role. A mutant form of PAI-1 that is unable to inhibit uPA also prevented cell migration by disrupting the interactions of VN and integrins.⁽⁷²⁾ Smooth muscle cell migration can also be inhibited in the presence of PAI-1 through the disruption of VN binding to the cell.⁽⁸⁷⁾

Apoptosis and the gene expression profile of PAI-1-expressing cells

Components of the PA system may regulate apoptosis. PAI-1 has been shown to inhibit apoptosis when added exogenously to both normal and tumor cells, again, due to its inhibition of uPA.⁽⁵⁵⁾ This fact alone might explain the PAI-1 paradox. Excess PAI-1 protects cancer cells from apoptosis. As a result, PAI-1 could promote the formation of a more aggressive tumor. A closer examination of PAI-1's inhibition of apoptosis showed that PAI-1 inhibits caspase-3-mediated apoptosis in vascular smooth muscle cells.⁽⁵⁶⁾

Slower proliferating cells tend to have a decreased sensitivity to chemotherapeutic agents.⁽⁸⁹⁾ We believe that endogenously produced PAI-1 confers a “survival advantage” upon cancer cells. It has been shown that the disruption of the cytoskeleton with chemotherapeutic drugs will alter the balance of protease-to-protease inhibitor in favor of PAI-1.⁽⁹⁰⁾ PAI-1 expression is affected by the perturbation of the actin cytoskeleton by cytochalasin D.^(57,58) Although multiple chemotherapeutic drugs are used to treat breast cancer, paclitaxel (Taxol) has shown efficacy in patients with drug resistance cancers, including breast cancer.⁽⁹¹⁾ In our studies with the PAI-1-expressing MDA-MB-435 cells, paclitaxel initially induces cell death in both wt-PAI-1-expressing and P14-PAI-1-expressing MDA-MB-435 cells (data not shown). However, when the cells were allowed to recover for 48 hours after treatment in complete medium, wt-PAI-1-expressing MDA-MB-435 cells had an increase in survival compared to P14-PAI-1-expressing MDA-MB-435 cells, although when plated onto either vitronectin or fibronectin surfaces the increase in survival between cell types was less different (data not included). These results imply that PAI-1 increases cell survival that depends on the integrity of the inhibitory site.

The upregulation of PAI-1 in a cancer cell is clearly conducive to promoting tumor cell progression to a malignant state. A cDNA microarray analysis was done on RNA extracted from the PAI-1-expressing MDA-MB-435 cells compared to the parental and vector control MDA-MB-435 cells. Of those genes, a list was generated of genes that were upregulated 2.5 or downregulated 0.5 compared to the MDA-MB-435 Neo cells (Table 2). Of the 23 genes upregulated in the wt-PAI-1-expressing MDA-MB-435 cells, many are associated with adhesion, motility and angiogenesis, thereby bringing nutrients to the tumor and providing a means for metastasis to occur. PAI-1 expression reduces cell proliferation by both upregulating genes associated with senescence (G protein-coupled receptor 1) and downregulating genes associated with promoting proliferation. These results agree with what has been published about the effects of PAI-1 on cancer.

PAI-1 expression in the MDA-MB-435 cells increases the expression of genes that are typically upregulated in various cancers, including breast cancer (Table 2). ELAV results in unstable mRNA in breast cancer⁽⁹²⁾ and eta polypeptide, a 14-3-3 protein involved in various signal transduction pathways, binds to gremlin 1, which is upregulated in numerous cancers, including breast cancer.⁽⁹³⁾

Two genes that were upregulated in the wt-PAI-1-expressing MDA-MB-435 cells would make the cells more responsive to stimuli produced by the MetS (Table 2). Protein phosphatase 2C, magnesium-dependent, catalytic subunit is a mitochondrial enzyme regulated by insulin.⁽⁹⁴⁾ Niemann-Pick disease, type C1 is a lysosomal membrane protein involved in cholesterol trafficking,⁽⁹⁵⁾ and, most importantly, in drug resistance by its drug efflux and sequestration.⁽⁹⁶⁾

The genes downregulated in the wt-PAI-1-expressing MDA-MB-435 cells were less numerous (Table 2). These genes are associated with proliferation, differentiation and adhesion. Apolipoprotein D regulates both proliferation⁽⁹⁷⁾ and cell migration.⁽⁹⁸⁾ Follistatin is typically downregulated in cancer and is an inhibitor of anti-proliferative pathways.⁽⁹⁹⁾ G

protein-coupled receptor 126, which is associated with adhesion,⁽¹⁰⁰⁾ is also downregulated by PAI-1 expression.

Conclusion

“What is food to one man may be fierce poison to others.” Lucretius (99 B.C.-55 BC).

Obesity is a worldwide problem that contributes to the risk and prognosis of many cancers, including breast cancer. With a high-caloric and high-fat Western diet, the resulting MetS will enhance the synthesis of PAI-1 and other components of the PA system in the tumor microenvironment. Adipocytokines (insulin, insulin-like growth factor 1, tumor necrosis factor- α , interleukin-6 and leptin/adiponectin) and other by-products of MetS (such as glucose and cholesterol) will alter PAI-1 expression not only in breast tissue adipocytes and in endothelial cells but also in breast cancer cells to potentially favor invasion and metastasis. The complex role and interaction between obesity and MetS linked to PAI-1 expression will clearly be studied for many years to come, as many study the pathological consequences of increased levels of PAI-1.

We hypothesize that increased expression of PAI-1 and other components of the plasminogen activator system (uPA and uPAR) confers a survival advantage upon breast cancer cells by decreasing sensitivity to chemotherapeutic agents, regulating adhesion, increasing tumor angiogenesis and increasing cell migration (Figs 1,2). As many researchers and clinicians have been and are still trying to fully understand the role of PAI-1 in cancer, the PAI-1 cycle hypothesis may further explain the paradox as to why a protease inhibitor of ECM degradation is detrimental for women with breast cancer (Fig. 2).

Acknowledgments

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Abbreviations

ECM	extracellular matrix
LRP	lipoprotein receptor-related protein
MMP	matrix metalloprotease
MetS	Metabolic Syndrome
P14 T333R PAI-1 MDA-MB-435	breast cancer cell line, MDA-MB-435, expressing a mutant PAI-1, which has a Thr replaced at position 333 with an Arg
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor-1
PAI-1^{-/-}	PAI-1 knockout
RAP	receptor-associated protein

uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
tPA	tissue-type plasminogen activator
VN	vitronectin
wt-PAI-1	wild-type PAI-1

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Figure 1.
Breast cancer, MetS and the PAI-1 cycle.

Figure 2.

Metastatic breast cancer and the PAI-1 cycle. Adipocytokines as a consequence of MetS will increase PAI-1 expression in breast adipocytes and surrounding tumor microenvironment. The increased levels of PAI-1 will support tumor angiogenesis, promote tumor cell adhesion/de-adhesion and activate signaling pathways to promote tumor cell invasion. PAI-1 will promote the generation of procoagulant microparticles from endothelial cells and thrombin will further promote PAI-1 synthesis. Fibrin will coat the tumor cells to help the tumor cells avoid immune surveillance. PAI-1 will also counteract apoptosis to provide a chemotherapy-resistant breast tumor microenvironment. Ultimately, the PAI-1 cycle will lead to sustained PAI-1 synthesis.

Table 1Stable Expression of wt-PAI-1 Decreases MDA-MB-435 Cell Proliferation^a

MDA-MB-435 Cell type	Anchorage-dependent proliferation (% CFE)	Anchorage-independent proliferation (%CFE)
Parental	100 ± 5	100 ± 6
Neomycin vector control	112 ± 19	90 ± 8
wt-PAI-1-expressing	53 ± 23 ^{**}	58 ± 14 [*]
P14-PAI-1-expressing	121 ± 9	113 ± 10

^aMDA-MB-435 cells PAI-1-expressing [wildtype (wt) and inactive T333R (P14)] and control MDA-MB-435 cells were generated as described previously.^{73,75,76} For anchorage-dependent proliferation, cells were plated at 50 cells per 60 mm² dish in 3 ml growth media and grown for 14 days. Colonies were fixed in 3:1 methanol:acetic acid and stained with Giemsa. Colonies of >50 cells were counted and expressed as a % of the number of colonies in the parental MDA-MB-435 population to yield colony-forming efficiency (CFE). For anchorage-independent proliferation, soft agar assays were performed by plating 20,000 cells per 60 mm² dish. Cells were suspended in a thin layer of 0.33% noble agar, which was overlaid on a 0.5% agar layer containing 10% FBS in 2× MEM. CFE's were assessed after 3 weeks by counting colonies with >50 cells. The data represent the average of 4 experiments performed in triplicate.

*
p < 0.05

**
p < 0.01, compared with the parental MDA-MB-435 cells.

Table 2

The Genetic Profile of wt-PAI-1-expressing MDA-MB-435 Cells

Genes upregulated ^d					Function
Wt-PAI-1-expressing MDA-MB-435:MDA-MB-435 Neo	MDA-MB-435:MDA-MB-435 Neo	UG Cluster	Gene Symbol	Gene	
18.63	1.22	Hs.414795	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (PAI-1)	Serine protease inhibitor; PAI-1
5.67	0.96	Hs.12379	ELAVL1	ELAV	RNA transporter/stabilizer; upregulation results in unstable mRNA in breast cancer
4.45	0.71	Hs.149609	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Adhesion/cell surface mediated signaling; interacts with uPAR; IGF-1 decreases its adhesion strength in breast cancer
4.08	1.23	Hs.414332	OAS2	2'-5'-oligoadenylate synthase 2, 69/71 kDa	Antiviral activity of interferons
3.77	0.86	Hs.194710	GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type	Inflammation/immune system
3.64	0.83	Hs.201641	BASP1	brain abundant, membrane attached signal protein	Regulate actin cytoskeleton/neuronal outgrowth in mammary gland
3.62	1.38	Hs.111779	SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	Extracellular matrix, adhesion; increased expression in breast cancer; also associated with increased cell motility
3.52	1.16	Hs.519385	FOXDI	forkhead box D1	Involved in the increase in PAI-1 gene expression
3.5	1.19	Hs.173894	CSF1	colony stimulating factor 1 (macrophages)	Regulate differentiation, activity, survival of osteoclasts and monocytes/macrophages; role in monocyte/macrophage mediated tumor angiogenesis
3.15	0.97	Hs.22265	PPM2C	protein phosphatase 2C, magnesium - dependent, catalytic subunit	Mitochondrial enzyme regulated by insulin
2.96	0.87	Hs.262960	TRPC4	transient receptor potential cation channel, subfamily C, member 4	Component of Ca2+ signaling pathway; activated by EGFR stimulation
2.96	0.86	Hs.528634	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	Antiviral activity of interferons
2.94	0.96	Hs.525392	SYNE2	spectrin repeat containing, nuclear envelope 2	Nuclear envelope scaffolding
2.91	0.95	Hs.517601	RAC2	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	Ras superfamily GTPase involved in cell growth, cytoskeletal reorganization, and protein kinase activation
2.83	1.27	Hs.78068	CPZ	carboxypeptidase Z	Member of metallocarboxypeptidase gene family; associated with extracellular matrix and Wnt binding
2.73	0.83	Hs.184907	GPR1	G protein-coupled receptor 1	Senescence associated gene
2.72	0.86	Hs.464779	NPC1	Niemann-Pick disease, type C1	Lysosomal membrane protein involved in cholesterol trafficking associated with drug clearance in the cell
2.66	0.97	Hs.468410	EPAS1	endothelial PAS domain protein 1	Hypoxia induced protein involved in angiogenesis; regulates PAI-1 and VEGF gene expression

Genes upregulated ^d					
Wt-PAL1-expressing MDA-MB-435:MDA-MB-435 Neo	MDA-MB-435:MDA-MB-435 Neo	UG Cluster	Gene Symbol	Gene	Function
2.65	0.87	Hs.249125	TLX3	T-cell Leukemia, homeobox 3	Involved in autonomic nervous system development; gene rearrangement (t(5;14)) upregulated in acute lymphoblastic leukemia
2.63	0.95	Hs.144795	KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	Component of a conductance, voltage, and Ca2 ⁺ sensitive channel
2.61	1.36	Hs.13155	ITGB5	integrin, beta 5	Adhesion to extracellular matrix; cell migration
2.58	0.79	Hs.474751	MYH9	myosin, heavy polypeptide 9, non-muscle	Cytoskeleton and cell migration
2.57	1.24	Hs.226755	YWHAH	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	14-3-3 protein involved in signal transduction; found to be bound to gremlin 1, which is upregulated in numerous carcinomas including breast
0.29	0.85	Hs.522555	APOD	apolipoprotein D	Regulate both proliferation and cell migration in response to growth factors
0.41	1.04	Hs.318894	GPR126	G protein-coupled receptor 126	G-protein coupled receptor involved in cell adhesion
0.42	1.14	HS.9914	FST	folistatin	Downregulated in cancer; inhibits activin, a member of the TGF-beta superfamily involved in regulating proliferation
0.44	1.39	Hs.25597	ELOVL1	elongation of very long chain fatty acids (FEN/Elo2, SUR4/Elo3, yeast)-like 2	Unknown
0.47	0.78	Hs.368226	SOX6	SRY (sex determining region Y)-box 6	Transcription factor involved in various developmental processes; upregulated in gliomas and not other cancers

^aThe MDA-MB-435 cell line was transfected using pcDNA3.1 vectors (Invitrogen) containing wt PAL-1 using Effectene (Qiagen) according to manufacturer's instructions. 73,75,76 Clones were selected for resistance to neomycin analogue, G418 (Invitrogen). In collaboration with the Microarray Core Facility at NHGRI, a cDNA microarray was used to compare the gene expression profiles among the parental MDA-MB-435, Neomycin vector control MDA-MB-435 (MDA-MB-435 Neo), and wt-PAL-1-expressing MDA-MB-435 cells. A standard human chip was used that contains over 29,000 elements: 28,200 human sequence verified clones and 800 controls. These genes represent over 24,400 unique Unigene clusters. One sample of RNA was labeled with Cy3 and the other with Cy5. The mixture of the two-labeled RNAs was competitively hybridized to a glass slide containing the cDNAs. For analysis, only genes in which the ratio between MDA-MB-435 and MDA-MB-435 Neo was between 0.7-1.4 were considered. From this list of genes the above tables were generated that list the genes upregulated 2.5 fold and those genes that are reduced by 0.5 compared to the MDA-MB-435 Neo cells.