The Serine Protease Inhibitor Protease Nexin-1 Controls Mammary Cancer Metastasis through LRP-1–Mediated MMP-9 Expression

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Abstract

Through their ability to degrade the extracellular matrix, proteases mediate cancer cell invasion and metastasis. Paradoxically, some serine protease inhibitors (serpins) are often overexpressed in human tumors. Using computational analysis, we found that the RNA level of protease nexin-1 (PN-1), a serpin that blocks numerous proteases activity, is significantly elevated in estrogen receptor-α-negative and in high-grade breast cancer. The *in silico* approach was complemented by mechanistic studies on two mammary cancer cell lines, the PN-1-negative 168FARN cells and the PN-1-positive 4T1 cells, both of which form primary mammary tumors, but only 4T1 tumors are able to metastasize to the lungs. We show that treatment of 168FARN cells with PN-1 stimulates extracellular signal-regulated kinase activation via low-density lipoprotein receptor-related protein-1 (LRP-1) binding, resulting in increased matrix metalloproteinase (MMP)-9 RNA, protein, and secreted activity. PN-1–silenced 4T1 cells express low MMP-9 levels. Moreover, injection of PN-1–silenced cells into mice did not affect 4T1 primary mammary tumor outgrowth; however, the tumors had impaired metastatic potential, which could be restored by reexpressing soluble MMP-9 in the PN-1–silenced 4T1 cells. Thus, using mammary tumor models, we describe a novel pathway whereby the serpin PN-1 by binding LRP-1 stimulates extracellular signal-regulated kinase signaling, MMP-9 expression, and metastatic spread of mammary tumors. Importantly, an analysis of 126 breast cancer patients revealed that those whose breast tumors had elevated PN-1 levels had a significantly higher probability to develop lung metastasis, but not metastasis to other sites, on relapse. These results suggest that PN-1 might become a prognostic marker in breast cancer. [Cancer Res 2009;69(14):5690–8]

Introduction

Disease recurrence and metastasis are the primary cause of cancer treatment failure and patient death. Metastasis is a multistep process involving different interrelated events (1). To escape the primary site and colonize secondary organs, tumor cells must move through and degrade surrounding tissue barriers. This process requires different cellular functions, an important one being proteolytic activity. Various types of proteolytic enzymes, including serine proteases (2) and matrix metalloproteinases (MMP; ref. 3), have important roles in the degradation of extracellular matrix components. The serine protease urokinase plasminogen activator (uPA) controls cell migration by promoting degradation of extracellular matrix components. Moreover, uPA plays an important role in the maturation of other proteins, including MMPs and growth factors, through its ability to convert plasminogen into plasmin (4). In human tumors, overexpression of uPA is a marker for poor clinical outcome (5, 6). Among the MMP family, the gelatinases MMP-9 and MMP-2 are of particular interest. Indeed, host-produced MMP-9 and MMP-2 have been shown to promote tumor-associated angiogenesis and metastasis (7). Moreover, tumor-produced MMP-9 has been associated with cancer progression (8).

Through their ability to reduce proteolysis, serine protease inhibitors (serpins), such as plasminogen activator inhibitor (PAI)-1 and -2, are predicted to impair extracellular matrix degradation and consequently cancer cell invasion and metastasis. Surprisingly, PAI-1 (SERPINE1), a serpin targeting uPA and tissue plasminogen activator (tPA), has been shown to promote angiogenesis and to induce tumor cell migration and invasion (9–11). Furthermore, clinical studies show that both uPA and PAI-1 are overexpressed in highly aggressive human breast tumors (6). Thus, results from clinical and cellular studies on PAI-1 are contrary to what would be expected for a protease inhibitor, observations that have led to the emergence of the "PAI-1 paradox" (12).

Protease nexin-1 (PN-1; SERPINE2), the phylogenetically closest relative of PAI-1, with a broader inhibitory role, blocks uPA, tPA, thrombin, factor XIa, prostasin, and trypsin activity (13–15). In a manner analogous to PAI-1, the covalent complex formed between PN-1 and its target proteases binds to the low-density lipoprotein receptor-related protein-1 (LRP-1), mediating internalization of the complex and ensuing degradation (16). Data on PN-1 expression in human cancer are sparse; however, studies on small sets of tumors suggest that PN-1 levels are elevated in pancreatic tumors (17), breast tumors (18), and oral squamous carcinomas (19). Furthermore, overexpression of PN-1 appears to enhance the invasive potential of pancreatic tumors in xenograft models (17). Although suggesting that PN-1 might also play a role in cancer progression, the precise function of this serpin in cancer still remains elusive.

We have used two mammary cancer cell lines that display different PN-1 expression patterns as models to study the role of PN-1 in breast cancer: the PN-1-negative 168FARN cells and the PN-1-positive 4T1 cells. Both cell lines rapidly form primary

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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mammary tumors when injected in fat pads of BALB/c mice, but only the 4T1 tumors metastasize to the lungs (20). We show that treatment of 168FARN cells with exogenous PN-1 induces MMP-9 RNA and protein expression, which is dependent on LRP-1 binding and extracellular signal-regulated kinase (ERK) activation. Moreover, short hairpin RNA (shRNA)-mediated PN-1 silencing in 4T1 cells causes a decrease in MMP-9 expression. Injection of PN-1–silenced 4T1 cells in mammary glands had no effect on outgrowth of primary mammary tumors; however, these tumors showed a dramatic impairment in their metastatic potential, which we show is due to the decrease in MMP-9 levels. Finally, in a microarray gene expression meta-analysis, we found that PN-1 levels are significantly increased in estrogen receptor (ER)-α-negative and in high-grade breast tumors. Furthermore, an analysis of 126 breast cancer patients revealed that those whose breast tumors had elevated PN-1 levels had a significantly higher probability to develop lung metastasis on relapse compared with metastasis to other sites. These results suggest that PN-1 might become a prognostic marker in breast cancer.

Materials and Methods

Reagents and antibodies. Mouse anti-PN-1 antibody (clone 4B3) was described previously (21). Rabbit anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-ERK1/2, anti-phospho-Akt (Ser^{473}), anti-Akt, anti-phospho-p38 mitogen-activated protein kinase (Thr^{180}/Tyr^{182}), anti-p38 mitogen-activated protein kinase, and anti-phospho-STAT3 (Tyr^{705}) were purchased from Cell Signaling. Anti-STAT3 was purchased from Transduction Labs. Rabbit anti-MMP-9 was from Abcam and monoclonal anti-actin was purchased from Chemicon. PN-1/protease complex inhibitory peptide (P960) and the control scramble peptide (P965), described in ref. 22, were purchased from Bachem. Human recombinant tPA and PAI-1 were purchased from Calbiochem, U0126 was from Catalysis, and bovine thrombin was from Chromogenix. Recombinant rat PN-1 was produced as described previously (23). Serpin/protease complexes were prepared by reacting tPA or thrombin with an equimolar concentration of the serpin for 10 min at 37°C. Oligonucleotides described in Supplementary Table S1 and small interfering RNAs were purchased at Microsynth.

Western and zymography analyses. Before collecting cell lysates for Western analysis or conditioned medium for zymography, 168FARN cells were starved in DMEM, and mouse embryonic fibroblasts (MEF) and 4T1 cells were starved in DMEM/1% stripped bovine serum albumin for 12 to 24 h. Immunoblots and zymographies were done as described previously (24).

Generation of PN-1-shRNA 4T1 clones and MMP-9 "rescued" clones. shRNAs were designed according to the Ambion Web site.[3] Two PN-1 mRNA target sequences were designed: PN-1-shRNA1 5′-AGAAACGGA-CATTCGTGGC-3′ and PN-1-shRNA2 5′-GCCGCGTACCTGTCACTAC-3′. shRNAs were cloned into pSilencer 1.0-U6 (Ambion) according to the manufacturer's instructions. 4T1 cells were cotransfected with the pSilencer PN-1-shRNA constructs and a vector encoding puromycin resistance using Lipofectamine 2000 (Invitrogen) as described (25). The empty pSilencer and puromycin vector were cotransfected to generate mock-transfected 4T1 cells. Clones that grew in DMEM/10% FCS with 20 μg/mL puromycin were isolated and examined for PN-1 levels by immunoblotting. To generate MMP-9 (or control) "rescued" 4T1 cells, two PN-1 knockdown (KD) clones (PN-1-shRNA1 clone 1 and PN-1-shRNA2 clone 1) were transfected with pcDNA-MMP-9 or with pcDNA. Two days post-transfection, stable pools were selected in medium containing 2 mg/mL neomycin.

Microarray gene expression analysis. The Oncomine Research Web site[4] was used to perform all the analyses of PN-1 expression across tumor data sets. In the Oncomine database, expression values are first $\log_2$ transformed, the median value per microarray is scaled to zero, and SD of values of each microarray is scaled to 1. We used P value cutoff of 0.05 (Student's t test) to select tumor data sets that displayed differentially expressed PN-1. Normalized $\log_2$ expression data of PN-1 were downloaded from the Oncomine Web site for further analyses.

For our analysis on PN-1 and metastasis, we used two publicly available data sets of patients (82 from ref. 26 and 58 from ref. 27). Primary breast tumor Affymetrix gene expression data were downloaded from Gene Expression Omnibus data repository[5]; relative accession numbers are reported in Results. The Affymetrix data were MAS5 normalized as described by the authors (26). We required at least a 5-year follow-up for patients without any metastasis, which resulted in the exclusion of 14 patients. Furthermore, we considered at least a >1.25-fold cutoff for PN-1-overexpressing primary breast tumors. Kaplan-Meier plots were generated by JMP IN software (SAS).

***In vivo* analyses of tumor and metastasis formation.** Animal experiments were done according to the Swiss laws governing animal experimentation and approved by the Swiss veterinary authorities. 4T1 cells (5×10^5) were injected in the fourth mammary fat pad of BALB/c mice (Charles River). For the experiment in Supplementary Fig. S7, parental 4T1 cells were injected into offspring of matings between *Pn-1* ± BALB/c mice. Once palpable, tumors were measured two to three times per week and size was calculated using the formula: height * [(diameter / 2)^2 * π]. On day 26, mice were sacrificed and tumors and lungs were dissected. Lungs were placed in Bouin's solution to visualize and count metastases.

A description of additional methods is provided in Supplementary Data.

Results

Differential PN-1 expression in mammary tumor cell lines. To investigate the role of PN-1 in cancer, we used the mammary carcinoma cell lines 168FARN and 4T1, which were isolated from a spontaneous BALB/c mammary tumor (20). Although both cell lines give rise to primary tumors following injection in the mammary fat pad, their invasive and metastatic behaviors differ (28, 29). Indeed, 168FARN cells only disseminate from primary tumors to the lymph nodes (28), whereas the 4T1 cells form tumors that metastasize to multiple sites including lungs (28, 29). PN-1 protein was not detected in 168FARN cells but was high in 4T1 cells (Fig. 1*A, top*). Thus, these cell lines represent interesting models to study the role of PN-1 in metastasis. PN-1 was shown previously to promote invasion of pancreatic cancer cells (17). Thus, we monitored activity of the secreted gelatinases MMP-9 and MMP-2, two crucial players in invasion, using zymography done on conditioned medium from these cells. 168FARN cells exhibit low MMP-9 and MMP-2 activity, whereas 4T1 cells have comparatively higher MMP-9 and lower MMP-2 activity (Fig. 1*A, bottom*).

To determine whether a link exists between PN-1 and gelatinase activity, the PN-1-negative 168FARN cells were treated with recombinant PN-1 and secreted gelatinase activity was measured 24 h later. PN-1 (0.2 μmol/L) induced an increase in MMP-9 activity without changing MMP-2 activity (Fig. 1*B*). Immunoblot analysis on conditioned medium of PN-1-treated 168FARN cells also revealed an increase in secreted MMP-9 (Supplementary Fig. S1). MMP-9 RNA levels were monitored by real-time PCR revealing that PN-1 caused a >40% increase of MMP-9 RNA, evident after 4 h of treatment (Fig. 1*C, top*). A Western analysis showed that MMP-9 and pro-MMP-9 protein levels increased with similar kinetics (Fig. 1*C, bottom*). It is worth mentioning that the conditioned medium of 168FARN and 4T1 cells reveals one band of MMP-9

[3] http://www.ambion.com
[4] http://www.oncomine.org
[5] http://www.ncbi.nlm.nih.gov/geo/

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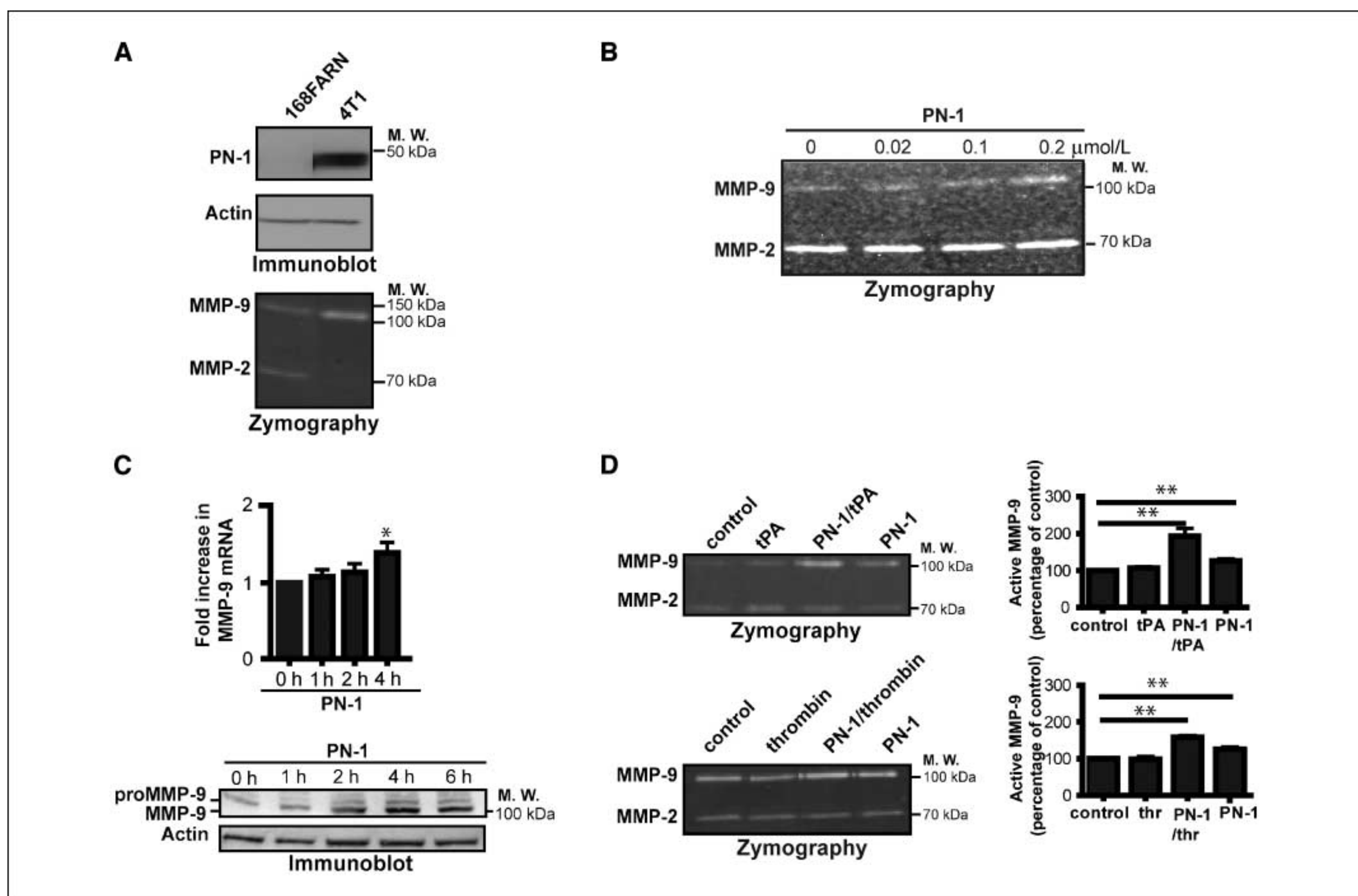

Figure 1. PN-1 induces MMP-9 expression. *A, top,* immunoblot analysis of PN-1 in 168FARN and 4T1 cells. Actin is used as control. *Bottom,* MMP-9 and MMP-2 activity examined by gelatin zymography on conditioned medium from 168FARN and 4T1 cells. *B,* gelatin zymography done on conditioned medium from 168FARN cells treated for 24 h with the indicated concentrations of recombinant PN-1. *C,* 168FARN cells were treated with 0.2 μmol/L PN-1 for the indicated times; RNA and cell lysates were prepared and real-time PCR (*top*) and Western analysis (*bottom*) for MMP-9 were carried out. Mean ± SD of triplicates. Representative of two independent experiments. *D,* gelatin zymography done on conditioned medium from 168FARN cells treated for 24 h with tPA (0.2 μmol/L), PN-1/tPA complexes (0.2 μmol/L), or PN-1 (0.2 μmol/L; *top*) and thrombin (*thr*; 0.2 μmol/L), PN-1/thrombin complexes (0.2 μmol/L), or PN-1 (0.2 μmol/L; *bottom*). *Columns,* ratio of MMP-9/MMP-2 activity. Mean ± SD of zymographic quantifications. *, $P < 0.05$; **, $P < 0.01$.

activity, which represents the active form of the gelatinase (data not shown). In summary, PN-1 treatment of 168FARN cells causes an increase in MMP-9 RNA and protein resulting in an increase in secreted active MMP-9.

Effects on MMP-9 expression are enhanced when PN-1 is complexed with a target protease. Serpins are generally complexed with their target proteases; indeed, high levels of serpin/protease complexes are found in many types of human cancer (30, 31). Thus, we examined the effect of PN-1/protease complexes on MMP-9 activity. 168FARN cells were incubated with PN-1 or tPA alone or with PN-1/tPA complexes and zymography was done on conditioned medium. Quantification revealed that tPA had essentially no effect. PN-1 caused a significant 20% increase in activity, whereas the PN-1/tPA complex induced an 85% increase in MMP-9 activity (Fig. 1*D, top*). Thrombin was also tested and yielded comparable results. The PN-1/thrombin complex was more efficient than PN-1 alone, causing a 50% increase in released MMP-9 activity (Fig. 1*D, bottom*). Thus, the stimulatory effect of PN-1 on MMP-9 activity is more pronounced when the serpin is complexed with target proteases.

LRP-1 mediates PN-1 effects. LRP-1 is a scavenger receptor responsible for serpin/protease catabolism. LRP-1 binds PN-1 with low affinity, whereas PN-1/proteases complexes show high affinity binding (32, 22). In the following experiments, we examined the role of LRP-1 in the PN-1 effects on MMP-9 expression.

Wild-type and LRP-1 knockout MEFs were treated with tPA, PN-1, or the PN-1/tPA complex and zymography was done on conditioned medium from the cells. In wild-type MEFs, there was an induction of MMP-9 activity, with the PN-1/tPA complex having the strongest effect (Fig. 2*A, top*), whereas, in LRP-1 knockout MEFs, none of the treatments stimulated MMP-9 activity (Fig. 2*A, bottom*). To substantiate the importance of the receptor in 168FARN cells, control and LRP-1-KD cells (Fig. 2*B*) were treated with the PN-1/tPA complex. MMP-9 activity was 40% lower in conditioned medium from LRP-1-KD cells compared with control cells (Fig. 2*C*). These results show the importance of LRP-1 in PN-1–mediated MMP-9 induction. In the final experiment, we tested the effect of P960, a 12-aminoacid peptide that was uncovered in a screen for peptides able to block PN-1/protease binding to LRP-1 (22). Treatment of 168FARN cells with 100 to 200 μg/mL P960 abrogated the ability of PN-1 to induce MMP-9 activity, whereas the control scramble peptide P965 had no effect on PN-1 activity (Fig. 2*D*). In conclusion, these results show that the recombinant PN-1 formed complexes with target proteases secreted

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by the 168FARN cells and it is this physiologically relevant PN-1/protease complex, via binding to LRP-1, that is responsible for MMP-9 induction.

Effects of PN-1 silencing in 4T1 tumor cells. In the following experiments, we examined the consequences of PN-1 down-regulation in 4T1 tumor cells. PN-1 was stably silenced by transfecting 4T1 cells with pSilencer vectors targeting two distinct sites in PN-1 mRNA (shRNA1 and shRNA2); transfection with the empty vector was done as a control. Following selection in puromycin-containing medium, PN-1-KD clones and control clones were isolated and PN-1 protein levels and secreted MMP-9 activity were examined. Clones with efficient shRNA1-mediated (Fig. 3*A, black columns, clones 1, 14, 29, 36*, and *37*) or shRNA2-mediated (Fig. 3*A, black columns, clones 1, 8*, and *34*) PN-1-KD showed a strong decrease in MMP-9 activity (Fig. 3*A, white columns*). Clones displaying little or no PN-1-KD (Fig. 3*A, black columns, shRNA1 clones 3* and *40* and *shRNA2 clones 14* and *36*) had essentially no change in secreted MMP-9 activity (Fig. 3*A, white columns*). These results support the link between PN-1 expression and MMP-9 activity that was established in the 168FARN cells. Two representative PN-1-KD clones, clone 1 shRNA1 and clone 1 shRNA2, were analyzed further. Zymography and immunoblot analyses show that both clones synthesized and secreted less MMP-9 than mock-transfected clones or untransfected 4T1 cells (Fig. 3*B*). Moreover, RT-PCR analysis revealed that, in comparison with control cells, MMP-9 mRNA was essentially undetectable in the PN-1-KD cells, whereas neither PAI-1 nor LRP-1 was altered in these cells (Fig. 3*C*).

PN-1 stimulates ERK signaling activity. Next, we examined the signaling pathways in control and PN-1-KD 4T1 cells. Parental and mock-transfected 4T1 cells have high levels of phospho-STAT3, phospho-p38 mitogen-activated protein kinase, and phospho-Akt, which remained unchanged in the two PN-1-KD clones (Fig. 4*A*). In contrast, compared with control cells, phospho-ERK1/2 levels were reduced in both PN-1-KD clones, the effect being most pronounced in PN-1-shRNA1 cells (Fig. 4*A*). To test if there is a link between ERK activity and MMP-9 expression, 4T1 cells were treated with the MEK inhibitor U0126 and MMP-9 protein levels were examined. Even the lowest dose of UO126 caused a >50% decrease in MMP-9 protein levels (Fig. 4*B*), suggesting that PN-1 signals via the ERK pathway to control MMP-9 expression in 4T1 cells.

Next, 168FARN cells were treated with recombinant PN-1 and ERK1/2 activity was evaluated. Within 15 min, PN-1 triggered an increase in ERK1/2 phosphorylation, which continued to increase and peaked at 30 min (Fig. 4*C*). This increase was more pronounced when 168FARN cells were incubated with PN-1/tPA complexes (Fig. 4*C, bottom*). To test if PN-1 stimulates MMP-9 expression via the ERK pathway, 168FARN cells were pretreated with the MEK inhibitor U0126 before PN-1 addition. UO126 pretreatment prevented PN-1 from enhancing MMP-9 levels (Fig. 4*D*), confirming the importance of ERK activation in the process.

PN-1 is required for 4T1 metastasis but not for primary tumor growth. Next, we tested the *in vivo* effect of PN-1 silencing in 4T1 cells. The two PN-1-KD clones (PN-1-shRNA1 clone 1 and PN-1-shRNA2 clone 1) as well as mock-transfected and parental 4T1 cells were injected into mammary fat pads of BALB/c mice and tumor formation was monitored. Over the course of 26 days, there was no significant difference in tumor outgrowth kinetics and tumor size between the PN-1-KD cells and the control 4T1 cells (Fig. 5*A*). The levels of PN-1 in the KD cells remained low throughout the experiment as revealed by a Western analysis

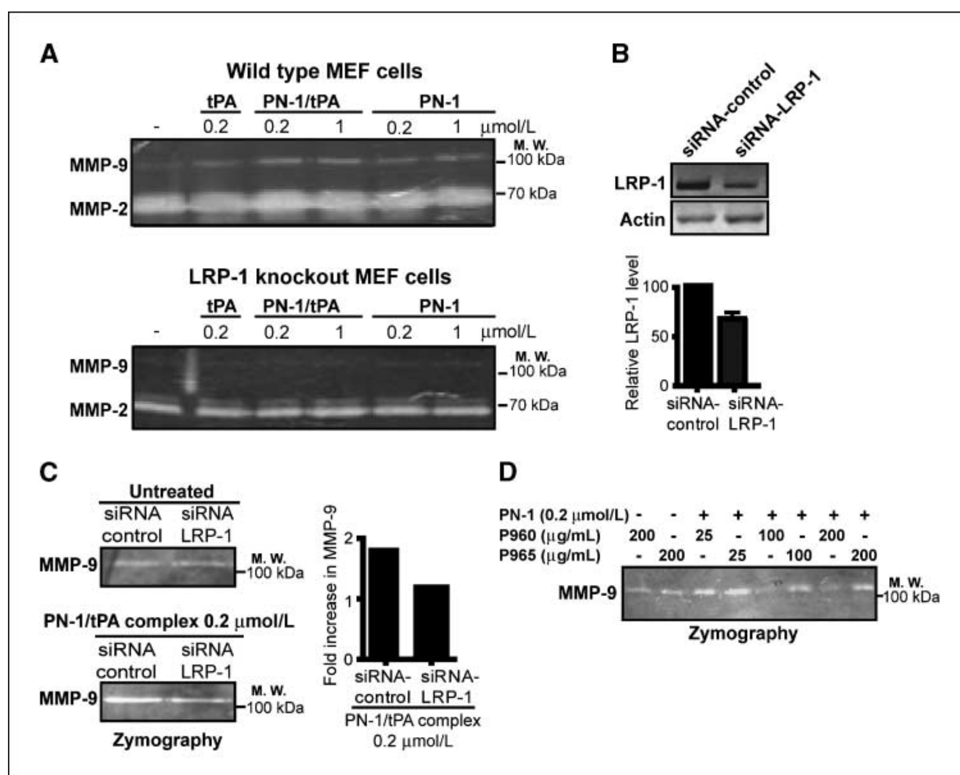

Figure 2. LRP-1 mediates the effect of PN-1. *A,* gelatin zymography done on conditioned medium from wild-type (*top*) and LRP-1 knockout (*bottom*) MEFs following treatment with tPA, PN-1/tPA complexes, and PN-1 at the indicated concentrations. *B,* semi-quantitative RT-PCR for LRP-1 in LRP-1-KD and control 168FARN cells 24 h post-transfection. *Columns,* relative LRP-1 levels in KD compared with control cells in three experiments. *C,* gelatin zymography analysis of LRP-1 and control small interfering RNA (*siRNA*)-transfected 168FARN cells treated or not with the PN-1/tPA complex (0.2 μmol/L) for 24 h. *Right,* quantification of MMP-9 activity by absorbance measurements of a gelatin zymography gel represented as fold increase in MMP-9 activity compared with the untreated small interfering RNA control. *D,* gelatin zymography done on conditioned medium from 168FARN cells incubated with the indicated concentrations of P960 inhibitory peptide or P965 scramble peptide with or without PN-1 for 24 h.

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carried out on lysates of tumors removed 26 days post-injection (Fig. 5*B*). To determine whether loss of PN-1 affects the metastatic ability of the 4T1 cells, the lungs of mice sacrificed on day 26 were stained with Bouin's fixative and the metastatic nodules were quantified. Whereas the tumors induced by parental and mock-transfected 4T1 cells formed multiple large nodules, tumors from the PN-1-KD cells formed very few metastases (Fig. 5*C*). Quantification revealed a significant 66% and 75% decrease in metastases resulting from PN-1-shRNA1 and shRNA2-KD tumors, respectively. These results show that PN-1, produced by the 4T1 tumor cells, is not essential for primary mammary tumor development. In contrast, the absence of PN-1 dramatically decreases the ability of 4T1 cells to disseminate to the lungs.

Expression of MMP-9 in PN-1-KD 4T1 cells rescues their metastatic potential. To assess if decreased MMP-9 expression in PN-1-KD 4T1 cells was responsible for their low metastatic properties, we generated MMP-9 "rescued" cells. A MMP-9 expression vector or a control empty vector was introduced into PN-1-shRNA1 and PN-1-shRNA2-KD 4T1 cells and stable pools were isolated. MMP-9 expression was restored in each PN-1-KD clone, with the PN-1-shRNA2 cells showing higher levels than the PN-1-shRNA1 cells (Fig. 5*D, top*). The MMP-9 "rescued" PN-1-KD cells as well as the control PN-1-KD cells were injected in mammary glands of BALB/c mice and tumor size and lung metastases were monitored. Tumor outgrowth of each of the four cell lines was equivalent to the outgrowth of the control (mock) 4T1 cells (Supplementary Fig. S2). Considering the metastatic potential of the cells, the control-rescued PN-1-KD cells formed significantly fewer metastases compared with 4T1 cells (mock; Fig. 5*D, bottom*), showing that the original PN-1-KD phenotype was maintained. Interestingly, PN-1-shRNA2-MMP-9 tumors gave rise to more metastasis than the 4T1 tumors (mock), whereas PN-1-shRNA1-MMP-9 tumors had the same number as control (Fig. 5*D, bottom*), perhaps reflecting the higher MMP-9 levels in the former cells (Fig. 5*D, top*). This experiment shows that ectopic MMP-9 expression in both PN-1-KD clones rescued their metastatic potential. Thus, we conclude that, in the 4T1 mammary tumor model, MMP-9 is responsible for PN-1–mediated metastasis.

In human breast tumors, PN-1 levels correlate with markers of poor prognosis and an increased chance of lung metastasis. There are only a few reports on PN-1 expression in human breast cancer (18), which prompted us to examine PN-1 levels in publicly available gene expression data sets (33). Our analysis shows that PN-1 expression increased in a statistically significant manner with breast tumor grade, and PN-1 levels were significantly higher in ER-α-negative tumors (Fig. 6*A*). The correlation between ER-α negativity and high PN-1 was validated in additional independent data sets encompassing >350 breast tumors (Supplementary Fig. S3). These results suggest that elevated PN-1 correlates with pathologic parameters predicting poor patient outcome.

To investigate the possibility that elevated PN-1 levels might have prognostic value, a cohort of breast cancer patients with complete clinical information (Gene Expression Omnibus accession nos. GSE2603 and GSE5327; ref. 26, 27) was analyzed. From the combined total of 126 patients, 38 developed metastases, of

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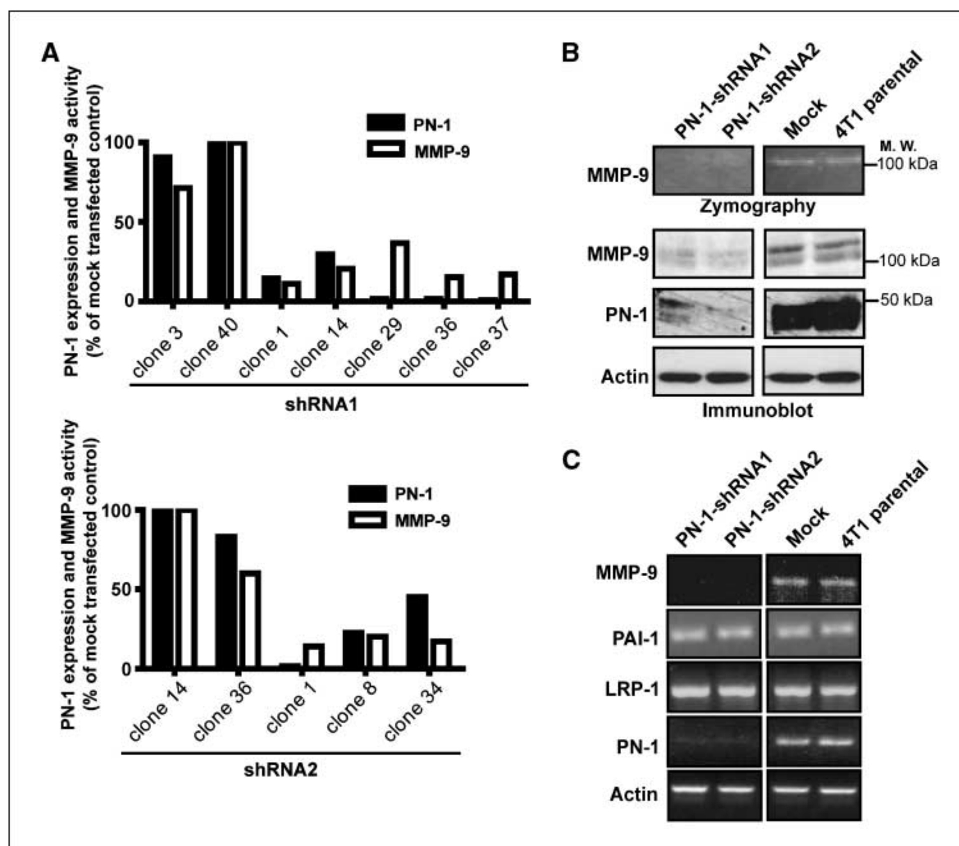

Figure 3. PN-1 silencing in 4T1 cells induces a decrease in MMP-9 expression. *A,* analysis of PN-1 levels and secreted MMP-9 activity in clones selected from shRNA1- and shRNA2-transfected 4T1 cells. PN-1 levels and MMP-9 activity were quantified by absorbance measurement of immunoblots and gelatin zymographies, respectively. *B,* MMP-9 activity analyzed by gelatin zymography done on conditioned medium and immunoblots done on cell lysates from a representative PN-1-shRNA1 4T1 and PN-1-shRNA2 4T1 clone and from mock and parental 4T1 cells. *C,* mRNA expression of MMP-9, PAI-1, LRP-1, PN-1, and actin (loading control) analyzed by semi-quantitative RT-PCR in PN-1-shRNA1, PN-1-shRNA2, mock, and parental 4T1 cells.

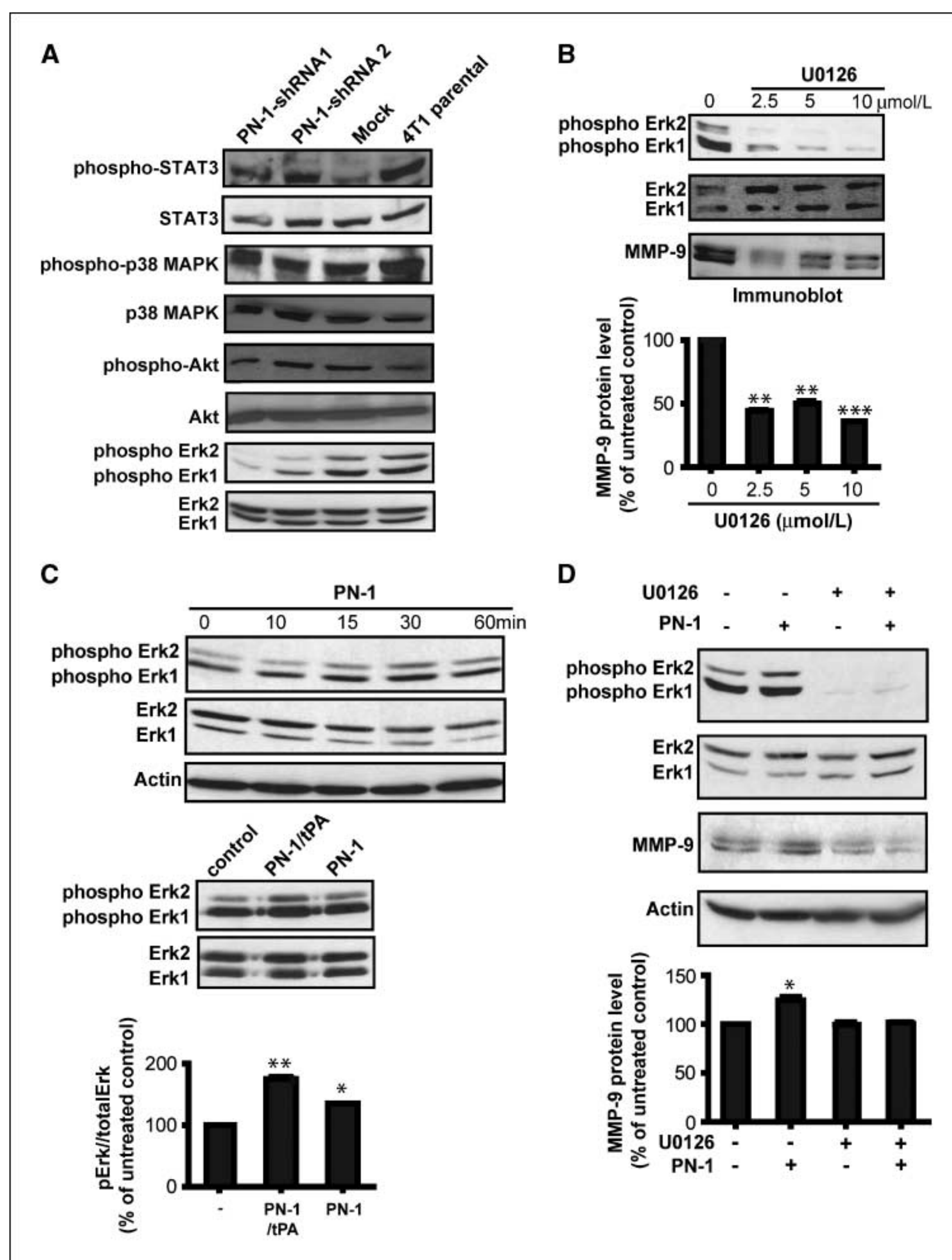

Figure 4. PN-1 mediates MMP-9 up-regulation through the ERK pathway. *A,* immunoblot for phospho-STAT3, phospho-p38 mitogen-activated protein kinase, phospho-Akt, and phospho-ERK1/2 in PN-1-shRNA1, PN-1-shRNA2, mock, and parental 4T1 cells; total level of each protein was also measured. *B,* immunoblot analysis of phospho-ERK1/2, total ERK1/2, and MMP-9 done on lysates of 4T1 cells treated for 12 h with the indicated concentrations of U0126. Mean ± SD of three different immunoblot quantifications. *C,* immunoblot for phospho-ERK1/2, total ERK1/2, and actin from lysates of 168FARN cells treated with 0.2 μmol/L PN-1 for the indicated times (*top*) and 0.2 μmol/L PN-1/tPA complex or 0.2 μmol/L PN-1 for 15 min (*bottom*). Mean ± SD of the ratio phospho-ERK/ERK from three immunoblot quantifications. *D,* immunoblot analysis of phospho-ERK1/2, total ERK1/2, MMP-9, and actin in lysates from 168FARN cells pretreated 30 min with 5 μmol/L U0126 followed by treatment with 0.2 μmol/L PN-1 for 15 min (ERK blots) and 6 h (MMP-9 blots). Mean ± SD of three different MMP-9 immunoblot quantifications. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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which 21 had lung metastases after a 5-year follow-up. Elevated PN-1 expression was significantly associated with lung metastasis probability (P = 0.039, log-rank test; Fig. 6*B*, *top*) but not with probability of metastasis at other sites (P = 0.26; Fig. 6*B*, *bottom*).

Discussion

Despite recent advances in breast cancer treatment (34, 35), the disease still remains a leading cause of death among women. The identification of novel pathways implicated in breast cancer formation or progression remains a high priority. The work we present here suggests that the serpin PN-1 and its receptor LRP-1 might be interesting targets. Using a combination of *in vitro* and *in vivo* experiments with mammary tumor models having distinct metastatic potential, we provide data supporting a role for PN-1 in cancer progression. We show that 4T1 mammary cancer cells require PN-1 to disseminate from the primary tumor to distant organs. Our results suggest a novel mechanism whereby PN-1, complexed with target proteases, binds the LRP-1 receptor and activates ERK signaling, thereby controlling MMP-9 expression and 4T1 metastatic spread (Supplementary Fig. S4). To expand our findings from mammary cancer models to human cancer, we examined publicly available expression data sets, which revealed that significantly higher levels of PN-1 are present in ER-α-negative and high-grade breast tumors. Furthermore, an analysis of 126 breast cancer patients revealed that those whose tumors have elevated PN-1 had a significantly higher probability

of showing lung metastasis on relapse. Results from this combination of approaches suggest that the PN-1/protease/LRP-1 complex might be a novel target for breast cancer therapy.

The LRP-1 receptor binds many proteins and functions as a signaling molecule in addition to its catabolic activity (36). We established the importance of LRP-1 in the PN-1/ERK/MMP-9 pathway that we describe here by various means. We show that PN-1 treatment of control MEFs, but not LRP-1-null MEFs, causes an increase MMP-9 expression. Similarly, LRP-1-KD 168FARN cells failed to produce MMP-9 in response to PN-1. These results rule out the possibility that the transmembrane glycoprotein syndecan-1, which has been shown to bind PN-1 and stimulate ERK activity in LRP-1-null MEFs (37), has a role in MMP-9 induction.

tPA (24) and PAI-1/uPA complexes (38) have also been shown to stimulate ERK activation through LRP-1. We have found that PAI-1/tPA addition to 168FARN cells also increases MMP-9 levels (Supplementary Fig. S5B), suggesting that these closely related serpins stimulate the same pathway, however, with some intriguing differences. Unlike PN-1 that stimulates MMP-9 when added alone to 168FARN cells, PAI-1 requires complexing with a protease (Supplementary Fig. S5A). The use of the P960 peptide, which only prevents PN-1/protease complexes from binding LRP-1, shows that recombinant PN-1 associates with proteases on the 168FARN cells and that these complexes stimulate MMP-9 expression. The reason why PAI-1 cannot complex with endogenous proteases on these cells is not known. Finally, it is interesting to mention that the 4T1 PN-1-KD cells maintain normal PAI-1 expression; however,

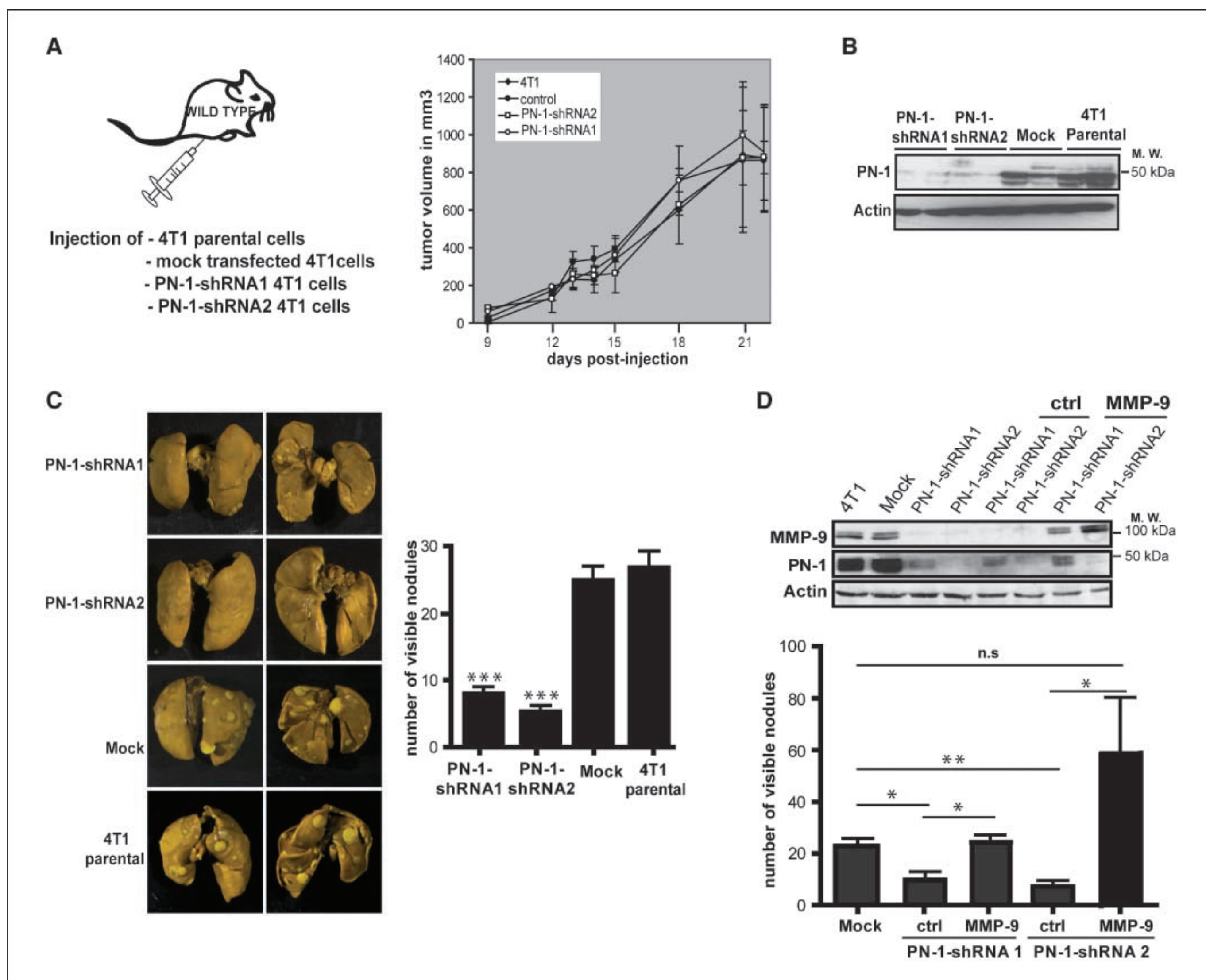

Figure 5. Expression of PN-1 in 4T1 cells is required for lung metastasis. *A,* injection of PN-1-shRNA1, PN-1-shRNA2, mock, and parental 4T1 cells in fat pads of BALB/c mice and growth curve of resulting primary tumors (n = 11 per group). *B,* immunoblot for PN-1 levels in PN-1-shRNA1, PN-1-shRNA2, mock, and parental 4T1 tumor lysates. Two tumors taken at day 26 were analyzed from each group. *C,* pictures of lungs from mice injected with PN-1-shRNA1, PN-1-shRNA2, mock, and parental 4T1 cells and quantification of lung metastases in each group (n = 11 per group). (Two views of the same lungs from a representative animal in each group are shown.) *D, top,* immunoblot for MMP-9 and PN-1 in PN-1-shRNA1, PN-1-shRNA2, pcDNA-transfected-PN-1-shRNA1 and -shRNA2 (*ctrl*), MMP-9-transfected-PN-1-shRNA1 and -shRNA2 (MMP-9), mock, and parental 4T1 cells. *Bottom,* quantification of lung metastases in mice bearing tumors arising from injection of pcDNA-transfected-PN-1-shRNA1 and -shRNA2 cells (*ctrl*), MMP-9-transfected-PN-1-shRNA1 and shRNA2 cells (MMP-9), and mock 4T1 cells (n = 5 per group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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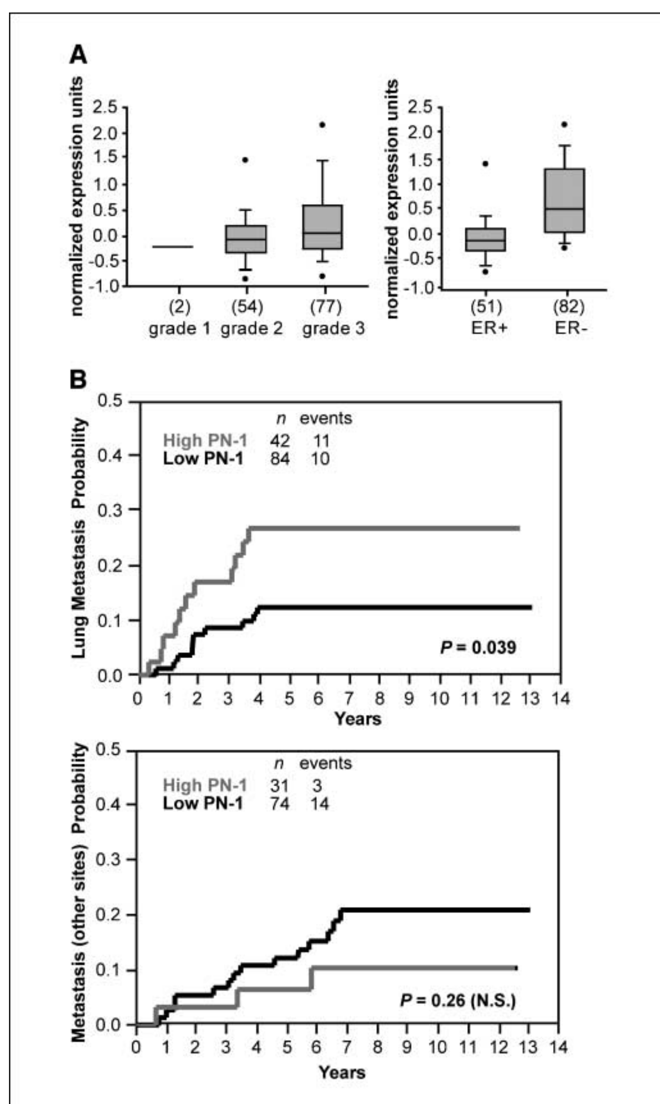

Figure 6. PN-1 in human breast tumors. Normalized PN-1 RNA levels from gene expression data sets in grades 1 to 3 and in ER-α-positive and ER-α-negative breast tumors. *P* values = 0.007 (*left*) and 3.70E-07 (*right*). *B,* Kaplan-Meier plots of 126 patients stratified by the expression level of PN-1. *Top,* probability of lung metastasis in patients with increased levels (>1.25-fold; *gray line*) and low levels (≤1.25-fold; *black line*) of PN-1. *Bottom,* probability of metastasis to other sites in patients with increased levels (>1.25-fold; *gray line*) and low levels (≤1.25-fold; *black line*) of PN-1. This analysis was done on the cohort, excluding patients with lung metastasis (21 patients).

this serpin cannot replace PN-1 function and promote MMP-9 expression in the 4T1 cells. The reason for this difference remains to be explored.

Our *in vivo* results with the 4T1 cancer model show that PN-1 is not required for mammary tumor outgrowth but has an essential role in metastasis. Loss of PN-1 causes a decrease of MMP-9 levels, which we show is the essential regulator of the metastatic phenotype. We have also established the importance of ERK signaling in PN-1–mediated MMP-9 expression, by showing that PN-1-KD in 4T1 cells causes a decrease in ERK activity and that pretreatment of 168FARN cells with a MEK inhibitor prevents PN-1 from stimulating MMP-9 expression. Down-regulation of the transcription factor Twist, which lies downstream of STAT3 in the 4T1 cells (39), has a similar phenotype as PN-1-KD. Specifically, Twist silencing does not prevent 4T1 mammary tumor outgrowth but decreases metastatic potential (29). PN-1-KD and control 4T1 cells have similar Twist mRNA levels (Supplementary Fig. S6), ruling out a role for Twist in the PN-1/LRP-1/ERK/MMP-9 pathway. MMP-9 has a well-documented role in metastasis in various cancer models (40). Based on our results showing that the re-expression of MMP-9 in the PN-1-KD 4T1 cells restores their metastatic potential, we propose that MMP-9 is the important target that mediates PN-1 effects on metastasis.

Serpins have multiple complex roles in tumor biology. Recently, host-derived PAI-1 was shown to be required for outgrowth of human neuroblastoma cells due to its ability to protect tumor-associated endothelial cells from apoptosis (11). PN-1 is expressed in many cell types including vascular endothelial cells (41) and stromal cells (42). We tested the role of host-derived PN-1 using PN-1-null mice (43) as recipients for 4T1 tumor cells. The outgrowth of primary tumors and appearance of lung metastases were similar in wild-type and PN-1 null mice (Supplementary Fig. S7). Taken together, our results show that host-derived PN-1 does not appear to have a role in 4T1 tumor outgrowth or metastasis, whereas tumor-derived PN-1 is essential for lung metastasis.

Our analysis of publicly available data sets revealed that PN-1 levels are significantly elevated in ER-α-negative tumors. Moreover, PN-1/SERPINE2 was recently reported to be one of the genes predicting poor outcome in breast cancer patients with ER-α-negative tumors (44). Using data from 126 breast cancer patients, we report here that elevated PN-1 expression was significantly associated with lung metastases probability on relapse but not with probability of metastasis at other sites. Taken together, these results support the hypothesis that, in breast cancer, elevated PN-1 levels might serve as a marker of poor prognosis and organ-specific metastatic potential.

Finally, it is worth mentioning that the LRP-1 receptor itself has a documented role in cancer. Indeed, silencing of LRP-1 in a breast cancer tumor model had no effect on primary tumor growth but decreased their metastatic potential (45). Furthermore, invasive properties of some breast and thyroid cancer cells appear to be dependent on LRP-1 (45, 46) and LRP-1 was shown to enhance gelatinase expression and migration of human glioblastoma cells (47). LRP-1 binds multiple serpin/protease complexes, and as we show here, PN-1/protease complexes activate signaling pathways and control expression of proteins with well-documented roles in cancer. Thus, targeting the LRP-1 receptor might be an appropriate therapeutic option.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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