Protease activated receptor-1 inhibits the Maspin tumor-suppressor gene to determine the melanoma metastatic phenotype

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The thrombin receptor protease activated receptor-1 (PAR-1) is overexpressed in metastatic melanoma cell lines and tumor specimens. Previously, we demonstrated a significant reduction in tumor growth and experimental lung metastasis after PAR-1 silencing via systemic delivery of siRNA encapsulated into nanoliposomes. Gene expression profiling identified a 40-fold increase in expression of Maspin in PAR-1–silenced metastatic melanoma cell lines. Maspin promoter activity was significantly increased after PAR-1 silencing, suggesting that PAR1 negatively regulates Maspin at the transcriptional level. ChIP analyses revealed that PAR-1 decreases binding of Ets-1 and c-Jun transcription factors to the Maspin promoter, both known to activate Maspin transcription. PAR-1 silencing did not affect Ets-1 or c-Jun expression; rather it resulted in increased expression of the chromatin remodeling complex CBP/p300, as well as decreased activity of the CBP/p300 inhibitor p38, resulting in increased binding of Ets-1 and c-Jun to the Maspin promoter and higher Maspin expression. Functionally, Maspin expression reduced the invasive capability of melanoma cells after PAR-1 silencing, which was abrogated after resucing with PAR-1. Furthermore, tumor growth and experimental lung metastasis was significantly decreased after expressing Maspin in a metastatic melanoma cell line. Moreover, silencing Maspin in PAR-1–silenced cells reverted the inhibition of tumor growth and experimental lung metastasis. Herein, we demonstrate a mechanism by which PAR-1 negatively regulates the expression of the Maspin tumor-suppressor gene in the acquisition of the metastatic melanoma phenotype, thus attributing an alternative function to PAR-1 other than coagulation.

The thrombin receptor is a seven-transmembrane G protein-coupled receptor also known as protease activated receptor-1 (PAR-1). PAR-1 activation leads to stimulation of G proteins triggering various downstream molecules and signal transduction pathways involved in cell growth, tumor progression, and metastasis of several cancer types including breast, prostate, and melanoma (1–3).

Our laboratory has found that PAR-1 is a key player in the progression of human melanoma, with overexpression of PAR-1 occurring in metastatic melanoma cell lines compared with nonmetastatic and primary melanoma cell lines (4). Furthermore, we and others have shown that melanoma tumors have increased PAR-1 expression compared with nevi (5, 6). Recently, we demonstrated that systemic delivery of PAR-1 siRNA encapsulated into neutral nanoliposomes inhibited tumor growth and experimental lung metastasis of melanoma cells in vivo (7). However, the exact mechanism by which PAR-1 contributes to melanoma growth, angiogenesis, and metastasis is not clear. By using gene expression profiling, we thus identified the serine protease inhibitor Maspin as a PAR-1 downstream target gene.

Maspin was first isolated from human mammary epithelial cells (8, 9). Its expression has been found to be decreased or lost in several malignancies and is associated with decreased aggressiveness by inhibiting tumor cell motility and invasion (10–12). Maspin expression has also been found to increase apoptosis as well as decrease angiogenesis in several cancers (13, 14).

In melanoma, Maspin was found to have a tumor-suppressor function. Denk and colleagues found that metastatic melanoma cells had decreased expression levels of Maspin compared with normal human epidermal melanocytes (15). Moreover, Maspin expression is lost with increased tumor thickness in clinical specimens as the tumor progresses from nonmetastatic to metastatic melanoma (16, 17). Previous reports have shown that early hypermethylation of the Maspin promoter might play a role in silencing Maspin. In addition, loss of Maspin was shown to correlate with increased tumor thickness (15–17).

Herein, we have identified an inverse correlation between PAR-1 and Maspin expression. PAR-1 differentially regulates the binding of c-Jun and Ets-1 transcription factors to the Maspin promoter through inhibition of phosphorylated p38 and activation of the histone acetyltransferase (HAT) CBP/p300, resulting in decreased Maspin expression. To our knowledge, this is the first report to identify PAR-1 as a regulator of the tumor suppressor Maspin, providing another mechanism by which PAR-1 contributes to the malignant phenotype of melanoma, and thus assigning a unique role for PAR-1 in tumor growth and metastasis in addition to its involvement in coagulation.

Results
Validation of Maspin as a Downstream Target Gene of PAR-1. Our group has previously demonstrated a significant decrease in tumor growth and experimental lung metastasis after silencing PAR-1 in metastatic melanoma cell lines by reducing angiogenic and invasive factors such as VEGF, IL-8, matrix metalloproteinase-2 (MMP-2), and Cx-43 (7, 18). To further investigate how PAR-1 contributes to the malignant phenotype, we sought to identify additional downstream PAR-1 target genes crucial to melanoma progression.

A375SM and C8161 metastatic melanoma cell lines were stably transduced with lentiviral-based PAR-1 shRNA. PAR-1 expression was reduced in both cell lines by more than 75% (Fig. 1 A and B and Fig. SI4). Although cDNA microarray analyses did not reveal a novel genetic program, several genes were regulated by PAR-1. Among them, Maspin expression was increased by more than 40-fold after PAR-1 silencing. Both protein and
mRNA levels of Maspin were significantly increased in PAR-1–silenced cell lines (Fig. 1C and Fig. S1B).

Transcriptional Regulation of Maspin After PAR-1 Silencing. To determine whether PAR-1 is a negative regulator of Maspin, the Maspin promoter was cloned in front of a luciferase reporter construct and transfected into nontargeting (NT) or PAR-1–silenced melanoma cell lines. The promoter activity of Maspin was significantly increased after PAR-1 silencing, suggesting that PAR-1 negatively regulates Maspin at the transcriptional level (Fig. S2).

Differential Binding of Ets-1 and c-Jun Transcription Factors to the Maspin Promoter. Maspin gene expression is regulated by several transcription factors, including AP-1 (Jun and Fos) and Ets-1 (19). Promoter mapping revealed two Ets-1 binding sites within 550 bp from the translation initiation site (TIS) as well as two AP-1 binding sites (Fig. 2A).

To determine how PAR-1 was regulating Maspin expression, we analyzed whether PAR-1 was affecting transcription factor binding to the Maspin promoter, as no significant differences in expression levels of total or phosphorylated Ets-1 or AP-1 were detected after PAR-1 silencing (Fig. S3A and B). ChIP studies revealed that silencing PAR-1 increased binding of Ets-1 and c-Jun to the Maspin promoter (Fig. 2B and C). However, binding of c-Fos was not detected in NT shRNA-transduced cells or PAR-1–silenced cells (Fig. S3C). These results suggest that PAR-1 affects Maspin expression through differential binding of both Ets-1 and c-Jun transcription factors.

Effects of Transcription Factor Binding-Site Mutations on Maspin Promoter Activity. To further analyze and corroborate that Maspin overexpression in PAR-1–silenced metastatic melanoma cells is a result of differential binding of Ets-1 and c-Jun, point mutations were made in both Ets-1 and AP-1 transcription factor binding sites.

As previously seen, Maspin promoter activity (without mutations) was again increased after PAR-1 silencing in both cell lines (Fig. S4). Mutating the proximal Ets-1 site completely abrogated these effects as Maspin promoter activity in PAR-1–silenced cells was similar to that in NT-transduced cells. Mutating AP-1 at position −54 also reduced Maspin expression in PAR-1–silenced cells but to a lesser degree than with the Ets-1 (−115) mutation in both cell lines. Interestingly, mutating the distal Ets-1 or AP-1 sites did not affect Maspin promoter activity in the C8161 cell line and minimally in A375SM as previously described (Fig. S4) (19). These results suggest that PAR-1 regulates Maspin promoter activity via the proximal Ets-1 site alone or in combination with the proximal c-Jun site.

PAR-1 Regulates Binding of Ets-1 and c-Jun to the Maspin Promoter Through CBP/p300. As no differences in Ets-1 or c-Jun protein levels were found after silencing PAR-1, we determined whether PAR-1 affected HAT activity, which could account for increased binding of Ets-1 and c-Jun to the Maspin promoter after PAR-1 silencing.

Previous studies have shown that PAR-1 activates the p38 MAPK pathway (20–23). A significant decrease in phospho p38 expression was found in PAR-1–silenced cell lines (Fig. 3A and Fig. S5). Total levels of p38 were not altered. Moreover, p38 activity inhibits the transcriptional coactivator CBP/p300 by decreasing its HAT activity (24, 25). In accordance with these reports, our studies revealed low levels of acetylated CBP/p300 in NT-transduced cells (high PAR-1, high phospho-p38 expressions) compared with PAR-1–silenced cells (Fig. 3B). By using an inhibitor of phospho-p38 (SB 203580) (26) (Fig. S6A), an increase in CBP/p300 expression is seen in NT-transduced cell lines, demonstrating that p38 inhibits CBP/p300 (Fig. 3B and Fig. S6B). Overall, these results suggest that PAR-1 inhibits CBP/p300 through increased p38 MAPK signaling.

Fig. 1. Maspin expression after PAR-1 silencing in A375SM and C8161 melanoma cell lines. FACS analyses reveal a significant decrease in PAR-1 expression in (A) A375SM and (B) C8161 cells after transduction with PAR-1 shRNA. Mouse IgG was used as an isotype control. As a negative control, secondary phycoerythrin (PE) ab was used without PAR-1 ab. Bar colors in histograms correspond to data from the representative FACS analysis image (inset). PE intensity indicates PAR-1 expression. (C) Western blot analysis of PAR-1–silenced cells depicts a significant increase in Maspin expression compared with NT-transduced cells. For all panels, data were obtained in triplicate and are expressed as mean values ± SD (*P < 0.001).

Fig. 2. Differential binding of Ets-1 and c-Jun transcription factors to the Maspin promoter. (A) Illustration of two AP-1 transcription factor binding sites (−510 and −54 from TIS) and two Ets-1 binding sites (−476 and −115 from TIS) within the first 550 bp of the Maspin promoter. (B) ChIP studies depict increased binding of Ets-1 and (C) c-Jun to the promoter of Maspin in both PAR-1–silenced cell lines compared with NT-transduced cells.

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To determine whether CBP/p300 expression directly affects Maspin expression, CBP/p300 siRNA was transduced in both metastatic cell lines (Fig. S6C). An increase in Maspin expression is seen after PAR-1 silencing, which is abrogated with the use of CBP/p300 siRNA (Fig. 3C and Fig. S6D). Moreover, inhibiting phospho p38 results in increased Maspin expression in NT shRNA-transduced cells (Fig. 3D and Fig. S6E), further establishing that PAR-1 regulates Maspin through increased p38 and decreased CBP/p300 expression.

CBP/p300 has been previously shown to bind directly to both c-Jun and Ets-1, thereby affecting their gene transactivation functions (27–29). Thus, we hypothesized that CBP/p300 overexpression, after PAR-1 silencing, increased binding of Ets-1 and c-Jun to the Maspin promoter. To test this hypothesis, ChIP analyses were performed with CBP/p300 siRNA. Silencing melanoma cells with both PAR-1 shRNA and CBP/p300 siRNA resulted in decreased transcription factor binding to the Maspin promoter compared with NT shRNA-transduced cells (Fig. 3E). These studies demonstrate that CBP/p300 is crucial for regulating binding of Ets-1 and c-Jun to the Maspin promoter.

Effects of PAR-1 Expression on Melanoma Cell Invasion. Previously, melanoma cell lines transfected with Maspin were found to have reduced invasive capacity (15). Invasion assays reveal that PAR-1 silenced melanoma cell lines had significantly lower numbers of invading cells (Fig. 4A). To determine that silencing PAR-1 results in decreased invasion through up-regulation of Maspin expression, PAR-1 was rescued in PAR-1 silenced cell lines (Fig. S7A). The protein levels of Maspin were significantly decreased after PAR-1 rescue, similar to Maspin protein levels from NT-transduced cells (Fig. 4B and Fig. S7B). To ascertain the inverse correlation between PAR-1 and Maspin, we used the SB-2 nonmetastatic melanoma cell line (i.e., low PAR-1 expressor) transduced with a PAR1 expression vector (30). Overexpression of PAR-1 in SB-2 cells also resulted in decreased expression of Maspin (Fig. S7C).

To corroborate that silencing PAR-1 results in decreased invasion through up-regulation of Maspin, PAR-1 rescued cells were subsequently used in invasion assays. Fig. 4C demonstrates that rescuing PAR-1 results in a significant increase in invasion. Furthermore, to determine whether the mechanism by which Maspin decreases the invasive capability of cells is a result of decreased MMP-2 activity, zymography assays were performed. In both A375SM and C8161 cell lines, PAR-1 silencing (i.e., high Maspin expression) resulted in decreased MMP-2 activity compared with NT-transduced cells (i.e., low Maspin expression; Fig. 4D). These effects were reversed when PAR-1 was rescued, showing that MMP-2 activity was higher in the PAR-1 rescued cells compared with PAR-1 silenced cells transduced with an empty vector (EV) construct (Fig. 4D).

To further demonstrate that Maspin was indeed affecting invasion, Maspin was overexpressed in both parental cell lines (i.e., low Maspin expression; Fig. S8A). These cells now showed decreased invasiveness (Fig. S8B), as previously reported (15). Moreover, a zymography assay was performed to determine whether Maspin...
of MMP-2 and VEGF (Fig. S10) that silencing Maspin in PAR-1 levels of NT-transduced cells. Furthermore, IHC analyses revealed tumor growth and experimental lung metastasis, although not to significantly reduced tumor growth and experimental lung metastasis (Fig. 5). 

Effects of Maspin Expression on Tumor Growth and Experimental Lung Metastasis. To determine the significance of Maspin in our in vivo model, we injected metastatic melanoma cell lines transduced with a Maspin expression vector, and found that overexpression of Maspin in A375SM significantly reduced tumor growth and experimental lung metastasis (Fig. 5 A and B). IHC analyses on tumors arising from cells transduced with a Maspin expression vector showed increased Maspin expression as well as decreased MMP-2 expression. Moreover, these Maspin-overexpressing tumors have decreased VEGF expression (Fig. S9). We have previously shown that PAR-1 can regulate expression of VEGF and MMP-2 in vivo (18). However, we now show that this can occur through regulation of Maspin by PAR-1.

To fully establish that PAR-1 inhibits the Maspin tumor-suppressor gene to promote the metastatic melanoma phenotype, Maspin was stably silenced in PAR-1–silenced cells (i.e., high Maspin expression; Fig. S10A). As seen in Fig. 5 C and D, silencing Maspin expression in PAR-1–silenced cells significantly increased melanoma tumor growth and experimental lung metastasis, although not to levels of NT-transduced cells. Furthermore, IHC analyses revealed that silencing Maspin in PAR-1–silenced cells increased expression of MMP-2 and VEGF (Fig. S10B).

Taken together, our data reveal a mechanism by which overexpression of PAR-1 contributes to the acquisition of the malignant phenotype of human melanoma by down-regulating the Maspin tumor-suppressor gene.

Discussion

Maspin has been previously described as having antiangiogenic and antimetastatic functions in several cancers (31, 32). In fact, receptor activator of NF-κB ligand-activated IκB kinase-α has been shown to translocate to the nucleus of prostate epithelial cells, resulting in inhibition of Maspin, thereby inducing prostate cancer metastasis to bone (33). Receptor activator of NF-κB ligand is known to regulate bone metastasis in prostate and breast cancers but does not seem to play a major role in melanoma metastasis (34–36). Although PAR-1 is known to activate the NF-κB pathway (37, 38), IκB kinase-α does not seem to function as a Maspin inhibitor in melanoma cells, again pointing to the tissue-specific regulation and function of Maspin.

Recently, the expression level of Maspin was found to be decreased in metastatic melanoma cells, which resulted in a more invasive phenotype (15). Increased tumor thickness also correlated with decreased Maspin expression, as only tumors samples less than 1 mm thick had positive Maspin expression (16, 17). Although Maspin promoter hypermethylation has been suggested to decrease Maspin expression (15, 17), we have found a link between PAR-1 and Maspin regulation through CBP/p300 HAT activity.

Metastatic melanoma cell lines were stably transduced with PAR-1 shRNA or NT shRNA and subjected to cDNA microarray analyses. Through such analyses, we have recently identified connexin 43 as a downstream target of PAR-1 (18). Herein, we describe PAR-1 regulation of Maspin in melanoma, which was found to be increased by more than 40-fold after PAR-1 silencing. These two regulatory events are not mutually exclusive, suggesting that PAR-1 is involved in several steps in the metastatic cascade including attachment to endothelial cells (Cx-43) and invasion (Maspin and MMP-2). Thus, the changes in the metastatic capacity of melanoma cells after PAR-1 silencing can occur through the regulation of several genes including, but not limited to, Cx-43, Maspin, MMP-2, VEGF, IL-8, and PAFR.

To further elucidate the role of Maspin in the metastatic phenotype of melanoma, we determined whether PAR-1 was regulating Maspin transcription. The Maspin promoter was thus cloned in front of a luciferase reporter vector. Our results indicate that Maspin promoter activity was increased by almost threefold in both A375SM and C8161 PAR-1–silenced melanoma cell lines. The significant increase in Maspin promoter luciferase activity demonstrates that PAR-1 regulates Maspin at the transcriptional level.

Although we did not find a difference in the protein expression of Ets-1 or AP-1 (Jun and Fos) after PAR-1 silencing, our ChIP analyses demonstrate an increase in both Ets-1 and c-Jun binding to the Maspin promoter in PAR-1–silenced cells. Interestingly,

**Fig. 4.** Effects of altering PAR-1 levels on Maspin expression and invasion. (A) Invasion assays demonstrate a significant decrease in the number of invasive PAR-1–silenced cells. Data are presented as means ± SD (⁎ P < 0.001). (B) Western blots revealed that Maspin is significantly reduced in PAR-1–rescued cells similar to Maspin protein levels from NT-transduced cells. (C) Rescuing PAR-1 significantly increased cell invasiveness compared with PAR-1–silenced cells transduced with EV (⁎ P < 0.001). Data are presented as means ± SD. (D) Zymography assay depicts decreased MMP-2 activity in PAR-1–silenced cells. Rescuing PAR-1 results in increased MMP-2 activity compared with PAR-1–silenced cells transduced with EV. FBS 1% is used as a positive control. Serum-free medium served as a negative control.
that re-expressing Maspin reduces the expression of MMP-2 and dioleoyl-phosphatidylcholine (7). Previous studies have also shown that these decreases in tumors from mice treated with PAR-1 siRNA previously demonstrated, through IHC analyses, that MMP-2 levels in causing Maspin promoter activation (19).

As PAR-1 did not affect the expression of either Ets-1 or c-Jun, we sought to elucidate the mechanism by which silencing PAR-1 resulted in increased binding of these transcription factors to the Maspin promoter. Previous reports demonstrated that p38 is activated by PAR-1 and that CBP/p300 HAT activity can be decreased by p38 (24, 25). Indeed, our results revealed a decrease in phospho-p38 along with an increase in CBP/p300 in PAR-1–silenced cells with a concomitant increase in Maspin expression. ChIP analyses show that inhibiting CBP/p300 decreased binding of Ets-1, c-Jun to the Maspin promoter. These data suggest that inhibiting CBP/p300 in PAR-1–silenced cells, thereby corroborating previous work demonstrating that CBP/p300 affected their transactivation capability (27, 28, 40, 41). It is important to note that PAR-1 does not affect expression of Ets-1 or AP-1. Rather, PAR-1 signaling increases binding of these transcriptional cofactors to the Maspin promoter. These data also suggest that the distance Ets-1 and c-Jun binding to the Maspin promoter is a result of decreased phospho-p38 levels and increased CBP/p300 expression.

Functionally, Maspin has been found to decrease the invasive capability of various cancer cell types, including melanoma. As PAR-1 did not affect the expression of either Ets-1 or AP-1, we sought to elucidate the mechanism by which silencing PAR-1 resulted in increased binding of the transcription factors to the Maspin promoter. Previous reports demonstrated that p38 is activated by PAR-1 and that CBP/p300 HAT activity can be decreased by p38 (24, 25). Indeed, our results revealed a decrease in phospho-p38 along with an increase in CBP/p300 in PAR-1–silenced cells with a concomitant increase in Maspin expression. ChIP analyses show that inhibiting CBP/p300 decreased binding of Ets-1 and c-Jun to the Maspin promoter. These data suggest that inhibiting CBP/p300 affects their transactivation capability (27, 28, 40, 41). It is important to note that PAR-1 does not affect expression of Ets-1 or AP-1. Rather, PAR-1 signaling increases binding of these transcriptional cofactors to the Maspin promoter. These data also suggest that silencing PAR-1 increases Ets-1 and c-Jun binding to the Maspin promoter as a result of decreased phospho-p38 levels and increased CBP/p300 expression.

Functionally, Maspin has been found to decrease the invasive capability of various cancer cell types, including melanoma. However, the decrease in invasion was abrogated when PAR-1 was rescued in PAR-1–silenced cells, which reverting to having low levels of Maspin similar to levels from NT-transduced cells.

This decrease in invasion in PAR-1–silenced, Maspin-expressing cells can be explained by a decrease in MMP-2 activity. We have previously demonstrated, through IHC analyses, that MMP-2 levels are decreased in tumors from mice treated with PAR-1 siRNA–dioleoyl-phosphatidylcholine (7). Previous studies have also shown that re-expressing Maspin reduces the expression of MMP-2 and the invasive capability of cells (15). Thus, silencing PAR-1 results in increased Maspin expression, ultimately leading to decreased cell invasiveness as a result of decreased MMP-2 activity.

Materials and Methods

ChIP. ChIP assays were performed by using the ChIP-IT Express kit from Active Motif according to the manufacturer’s protocol. The PCR for Maspin transcription factors (Ets-1, c-Fos, and c-Jun) was performed using the REDAccuTaq LA DNA Polymerase (Sigma) in a 25-μL reaction mixture containing 5% DMSO. A 662-bp fragment spanning −579 to +83 region of the Maspin pro-
motor was amplified by PCR by using 10 μM of primers 5'-GCGCTTGATCTCCTAATACAG-3' and 5'-CGGCTGACGAAGGTGCTGATCGTTCGACC-3'.

PAR-1 Rescue Expression Vector. PAR-1 constructs with an N-terminal prolactin signal peptide and flag tag (provided by Shaun R. Coughlin, University of California, San Francisco, CA) were combined with a nontargetable PAR-1 coding region and ligated into the pLVX-DSRed-Monomer-C1 vector; Clontech as previously described (18).

Invasion Assays. Invasion assays were performed using Biocoat Matrigel invasion chambers (Becton-Dickinson) primed according to the manufacturer's instructions. B20% in normal growth medium was placed in the lower chamber as a chemoattractant. Melanoma cells (2.5 × 10^5) in 500 μl of growth medium containing 0.2% FBS were added to the upper chamber of the Matrigel plate and incubated at 37 °C overnight. Cells on the lower surface of the filter were stained with Hema 3 staining kit (Fisher) and counted. Data were expressed as average number of cells from four fields per sample that migrated to the lower chamber. Triplicates were run per cell condition.

Zymography. MMP-2 activity was determined on substrate-impregnated gels as previously described (43).


In Vivo Experiments. In vivo experiments were performed as previously described (7) in accordance with an animal protocol approved by the M. D. Anderson Cancer Center Institution Animal Care and Use Committee. Briefly, 5 × 10^5 A375SM cells transduced with a Maspin expression vector or 2.5 × 10^5 A375SM cells transduced with NT shRNA alone or PAR-1 shRNA alone or in combination with Maspin shRNA were injected s.c. into nude mice. Levels were measured twice weekly and mice were killed at 5 wk. To analyze for experimental lung metastasis, 1 × 10^5 cells were injected i.v. into the tail vein of mice, which were killed after 5 wk, at which time the lungs were fixed in Bouin solution and the metastatic colonies counted (n = 10 per group).

Statistics. The Student t test was used to evaluate the data. P values less than 0.05 were considered statistically significant.

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Supporting Information

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SI Materials and Methods

Cell Lines and Culture Conditions. The A375SM human melanoma cell line was maintained in modified Eagle medium supplemented with 10% FBS, as previously described (1). The C8161 and SB-2 human melanoma cell lines were maintained in DMEM-F12 supplemented with 5% FBS, as previously described (1, 2). The 293FT cells (Invitrogen) used to make lentiviral shRNA were maintained in DMEM supplemented with 10% FBS, per manufacturer’s instructions.

Antibodies and Reagents. The PAR-1 antibody, ATAP2, used for FACS analyses was purchased from Santa Cruz Biotechnology. The PAR-1 antibody used for Western blot analyses was purchased from Immunotech Coulter. APC antibody was purchased from BioLegend. The PE (anti-mouse) antibody was purchased from Jackson ImmunoResearch. Anti-human Maspin antibody used for Western blots was purchased from BD Pharmingen. Ets-1, c-Jun, phospho-c-Jun, c-Fos, phospho-c-Fos, and IgG antibodies used for ChIP and Western blot assays were purchased from Santa Cruz Biotechnology. Phospho-p38, p38, and CBP/p300 antibodies were purchased from Cell Signaling Technology. Antibodies used for immunostaining include MMP-2 (Chemicon), Maspin (Abcam), VEGF (Santa Cruz Biotechnology), and goat anti-mouse or anti-rabbit HRP-IgG secondary antibodies (Jackson ImmunoResearch). The inhibitor of p38, SB 203580, was purchased from Calbiochem.

shRNA and siRNA. PAR-1 shRNA (target sequence, AGATTAG-TCTCCATCAATA) and Maspin shRNA (target sequence, GG-TGAGACTGCAATGGAAT), as well as an NT shRNA (target sequence, TTCTCCGAAACGGGTACGT), were used with the lentiviral system developed by Didier Trono as described previously (1). CBP/p300 siRNA (target sequence, AACCCTTCTCTCATTGACCCACA) was purchased from Dharmacon and transfected into PAR-1-silenced cells by using Hiperfect transfection reagent (Qiagen) per manufacturer’s instructions.

Flow Cytometry. Flow cytometry was performed as previously described (1).

Western Blot Analysis. Maspin (1:1,000), phospho-p38 (1:1,000), p38 (1:1,000), and PAR-1 (1:250) were detected in total cell extracts by 10% SDS-polyacrylamide gel electrophoresis as we previously described (1). Ets-1, c-Jun, phospho-c-Jun, c-Fos, and phospho-c-Fos (1:1,000) were detected in nuclear extracts by using the Nuclear Extraction Kit from Panomics per manufacturer’s instructions. CBP/p300 was detected in nuclear extracts by 8% SDS-polyacrylamide gel electrophoresis and transferred into an Immobilon-P transfer membrane (Millipore) using transfer buffer with 0.1% SDS overnight at 4 °C (30 V) followed by 1 h at room temperature at 100 V. The membranes were washed in Tris-buffered saline solution with Tween 20 (10 mm Tris-HCl, pH 8, 150 mm NaCl, and 0.05% Tween 20) and blocked with 5% nonfat milk in Tris-buffered saline solution with Tween 20 for 4 h. The blots were then probed overnight at 4 °C with primary antibody followed by 2 h of incubation with horseradish peroxide-conjugated secondary antibody. Immuno-reactive proteins were detected by enhanced chemiluminescence using the ECL detection system per manufacturer’s instructions (GE Healthcare). For Western blots using the p38 inhibitor, SB203580, cells were treated with 10 μM for 24 h before extracting protein, as previously described (3).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed as previously described (1) using the following Maspin specific primers: forward, 5′-GCTTTTGGCGCTTGATCGTTC-3′; reverse, 5′-GATCGACGTTCATTTCTCTC-3′.

cDNA Microarray. Microarray analysis was performed using the human Genome U133 Plus 2.0 Array (Affymetrix). The microarrays were produced in the microarray core facility of Codon Biosciences (Houston, TX). Total RNA was isolated from NT shRNA and PAR-1–silenced cells with the RNAqueous kit (Ambion) according to the manufacturer’s instructions. The data were deposited by Codon Biosciences. Raw data have been deposited in the Gene Expression Omnibus database (accession no. GSM596624).

Reporter Constructs and Luciferase Activity Assays. The Maspin promoter region (−1.1 kb to +83 bp from the transcription initiation site) was amplified from C8161 genomic DNA using the following primers: forward, 5′-GGGGTACCGACTGACATCTC-3′; and reverse, 5′-CCGCTAGGGCACGCTGGTGCTACCTG-3′. The fragment was digested with KpnI and XhoI and ligated into the pGL3-basic vector (Promega). Site-directed mutagenesis for Ets-1 elements was performed by two nucleotide substitutions (italic) in the Ets-1 binding motif (CTCCCT to CTTCCCT) to create mutations as described previously (4): Ets-1 (−476) 5′-GATTTTTAAAAAGAAACGTGCTGCGCACCATTAAAC-3′; Ets-1 (−115), 5′-GTA-ACTCA-CAGCCCCGTGTCGCCAAATCTGTGAGG-3′.

The AP-1 binding site at position −54 was mutated from TGAGTAA to GCGACTCA (5), whereas the AP-1 site at position −510 was mutated from TGAGTAA to GGACTAA as previously described (6, 7): AP1 (−510), 5′-GGCGCGCGCGGGGGGGCCAACTTACCCACAGG-3′; AP1 (−54), 5′-CAGTAGCTTCA-GCCACAAATTTCTTTCTCATTTG-3′.

Transient transfections of these constructs were performed using Lipofectin (Invitrogen) according to the manufacturer’s instructions. A total of 2.5 × 10⁴ cells per well in a 24-well plate were transfected with 0.5 μg of the pGL3-basic expression vector with no promoter or enhancer sequence, with 0.5 μg of pGL3-Maspin or pGL3-Maspin-mutant firefly luciferase expression constructs. For each transfection, 2.5 ng of CMV-driven renilla luciferase reporter construct (Promega) was included. After 6 h, the transfection medium was replaced with serum-containing growth medium. After 72 h, the cells were harvested and subjected to lysis, and the luciferase activity was assayed by using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. The luciferase luminescence (relative light intensity × 10⁶) was measured with the LUMItstar reader (BMG Labtech). The ratio of firefly luciferase activity to CMV-driven renilla luciferase activity was used to normalize for differences in transfection efficiency among samples.

Maspin Expression Vector. A 1.2-kb region of the Maspin ORF was amplified from C8161 genomic DNA using the following primers: forward, 5′-CATATGGATGCGCCTGAACTAGCAAATTC-3′; reverse, 5′-TTAAGGGAACAGAATTTGCTG-3′. The PCR product was performed using PfuUltra Hotstart PCR Master Mix (Stratagene). The PCR product was run on an agarose gel, the appropriate band was cut, and the DNA was purified by using the GeneClean Spin Kit (Biozyme). The purified Maspin DNA was cloned into pcDNA4/HisMax Topo expression vector (Invitrogen) according to the manufacturer’s protocol. For stable transduction of the Maspin expression vector, the insert was
digested from the pcDNA4 expression vector with MluI followed by blunt ending and a second digestion with XhoI. The DNA was recovered after running product on an agarose gel and purifying the DNA. The Maspin insert was then ligated into the pLVX-DsRed-Monomer-C1 vector using XhoI.

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded sections were deparaffinized by sequential washing with xylene and graded ethanol (100%, 95%, 80%) and rehydrated in PBS solution. Antigen retrieval was done by heating in a steam cooker in 1x Target Retrieval Solution (Dako) for 20 min. After cooling and washing with PBS solution, endogenous peroxide was blocked with 3% hydrogen peroxidase inhibitor in PBS solution for 12 min. Nonspecific proteins were blocked in 5% horse serum and 1% goat serum for 20 min. Slides were incubated with MMP-2 polyclonal antibody (1:400) and Maspin antibody (1:200) overnight at 4 °C. Slides were washed, incubated for 10 min in protein-blocking solution, and reacted with goat anti-mouse or goat anti-rabbit HRP-IgG secondary antibodies (1:500) for 1 h at room temperature. Signal was detected with 3,3′-diaminobenzidine (Phoenix Biotechnologies) substrate for 6 min and counterstained with Gill no. 3 hematoxylin (Sigma) for 20 s. VEGF was analyzed by immunohistochemistry as we previously described (1).


**Fig. S1.** Stable silencing of PAR-1 in metastatic melanoma cell lines and its effect on Maspin expression. (A) Western blot analyses demonstrate a significant decrease in PAR-1 protein expression after stably silencing two metastatic melanoma cell lines with PAR-1 shRNA. Data are presented as means ± SD from three independent experiments (*P* < 0.001). (B) Semiquantitative RT-PCR demonstrates an increase in Maspin mRNA levels after stably silencing PAR-1 in two metastatic melanoma cell lines.

**Fig. S2.** Transcriptional regulation of Maspin promoter in PAR-1-silenced metastatic melanoma cell lines. The luciferase activity driven by the Maspin promoter was significantly increased by PAR-1 silencing in both (A) A375SM and (B) C8161 cell lines compared with NT-transduced cells. The ratio of firefly luciferase activity to CMV-driven renilla luciferase activity was used to normalize for differences in transfection efficiency among samples (*P* < 0.001). Data are presented as means ± SD from three independent experiments.

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Fig. S3. Effects of inhibiting PAR-1 and phospho p38 on AP-1 and Ets-1 expression. No significant differences are seen in either phospho- or total protein levels of (A) Ets-1 or (B) c-Jun after PAR-1 silencing or after inhibiting phospho-p38 using SB 203580. Data are presented as means ± SD from three independent experiments. (C) ChIP studies failed to detect binding of c-Fos to the Maspin promoter in PAR-1-silenced cell lines or NT-transduced cells. Input DNA is used to determine equal amounts of chromatin in each condition used.
Fig. S4. Effects of Ets-1 and AP-1 transcription factor binding site mutations on Maspin promoter activity in PAR-1–silenced metastatic melanoma cell lines. Nonmutated Maspin promoter activity is increased after PAR-1 silencing in A375SM and C8161 melanoma cell lines (P < 0.001). Mutating the proximal Ets-1 site completely abrogated these effects in both cell lines. Mutating AP-1 at position −54 also reduced Maspin expression in PAR-1–silenced cells but to a lesser degree than with the Ets-1 (−115) mutation in both cell lines. The distal Ets-1 or AP-1 sites did not affect Maspin promoter activity in C8161 cell lines and minimally in A375SM. Data are presented as means ± SD from three independent experiments (**P < 0.05, *P < 0.001).

Fig. S5. Densitometry of phospho-p38 expression after PAR-1 silencing. A significant difference in phospho-p38 protein expression is seen by densitometry analysis after PAR-1 silencing in both metastatic melanoma cell lines (*P < 0.001). Data presented as mean ± SD from three independent experiments.
Fig. S6. Phospho-p38 and CBP/p300 regulate Maspin expression levels in metastatic melanoma cell lines. (A) Western blot analyses depict a significant decrease in phospho-p38 protein levels in PAR-1–silenced cells compared with NT shRNA-transduced cells. (*P < 0.01). Using the p38 inhibitor, SB 203580, NT shRNA-transduced cells now show decreased levels of phospho-p38 (*P < 0.001). (B) Densitometry analyses depict a significant increase in CBP/p300 protein levels after using the p38 inhibitor (SB 203580) in NT-transduced metastatic melanoma cell lines (*P < 0.001). (C) Western blot analyses depict a significant decrease in CBP/p300 protein levels after transfecting PAR-1–silenced cells (i.e., high CBP/p300 expressors) with CBP/p300 siRNA (*P < 0.001). (D) Densitometry from Western blot analyses depict a significant decrease in Maspin protein expression after using CBP/p300 siRNA on PAR-1–silenced cells (i.e., high Maspin expressors; **P < 0.01). (E) Increased Maspin expression is seen in NT shRNA-transduced cells after decreasing phospho-p38 using SB 203580 (*P < 0.001). Data from all experiments are presented as mean ± SD from three independent experiments.
Fig. S7. Re-expressing PAR-1 using a nontargetable PAR-1 expression vector in A375SM and C8161 metastatic human melanoma cell lines. (A) A375SM and C8161 melanoma cell lines were transduced with a nontargetable PAR-1 expression vector (seven silent point mutations are present in the ORF of the PAR-1 sequence targeted by the stably transduced PAR-1 shRNA). Both cell lines show an increase in PAR-1 expression after PAR-1 rescue (orange) compared with PAR-1–silenced cells transduced with an EV control (red). APC and PE intensity (Insets) indicate PAR-1 expression. Representative images from three independent experiments are shown. (B) Densitometry from Western blot depict a significant decrease in Maspin expression after PAR-1 is restored in PAR-1–silenced metastatic melanoma cell lines (*P < 0.001, **P < 0.01). (C) Western blot depicting a significant decrease in Maspin protein expression when PAR-1 is transduced into a low PAR-1–expressing nonmetastatic melanoma cell line, SB-2 (*P < 0.001). Data presented as mean ± SD from three independent experiments.
Fig. S8. Effects on invasion after expressing Maspin in metastatic melanoma cell lines. (A) Western blot depicts A375SM and C8161 metastatic melanoma cells transduced with a Maspin expression vector having significantly higher levels of Maspin protein expression compared with cells transduced with EV control ($P < 0.001$). (B) Maspin-transduced A375SM and C8161 cell lines had a significant decrease in invasive capability when using a Matrigel chamber compared with cells transduced with an EV control (*$P < 0.01$). Data are presented as means ± SD from three independent experiments. (C) Zymography assay depicts decreased MMP-2 activity in both A375SM and C8161 cells transduced with a Maspin expression vector compared with EV control (i.e., low Maspin expressors). FBS 1% is used as a positive control. As a negative control, serum-free medium was used.
In vivo effects of Maspin in melanoma. Tumors arising from A375SM metastatic melanoma cells stably transduced with Maspin or with an EV control were harvested and analyzed by IHC for Maspin and MMP-2 expression as well as VEGF. Tumors from mice injected with Maspin-expressing A375SM cells had increased Maspin expression along with decreased MMP-2 and VEGF levels. Images are shown at 20× magnification.
Fig. S10. Effects of silencing Maspin in PAR-1–silenced cells. (A) Western blot depicts a significant decrease in Maspin protein expression levels in PAR-1–silenced cells (i.e., high Maspin expressors) after stable transduction with Maspin shRNA in two metastatic melanoma cell lines (*P < 0.001). Data are presented as means ± SD from three independent experiments. (B) Tumors from A375SM metastatic melanoma cells stably transduced with NT shRNA, PAR-1 shRNA, or PAR-1 shRNA plus Maspin shRNA were harvested and analyzed by IHC. Tumors arising from A375SM transduced with PAR-1 shRNA and Maspin shRNA show increased MMP-2 and VEGF levels compared with PAR-1–silenced cells alone. Images are shown at 20× magnification.