Loss of Receptor Protein Tyrosine Phosphatase β/ζ (RPTP β/ζ) Promotes Prostate Cancer Metastasis*

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Background: The role of pleiotrophin and its receptors RPTP β/ζ and Syndecan-3 during tumor metastasis remains unknown.

Results: RPTP β/ζ knockdown initiates EMT, promotes pleiotrophin-mediated migration and attachment through Syndecan-3 and induces in vivo metastasis.

Conclusion: RPTP β/ζ plays a suppressor-like role in prostate cancer metastasis.

Significance: Boosting RPTP β/ζ or attenuating Syndecan-3 signaling pathways may lead to more effective therapeutic strategies in treating prostate cancer metastasis.

Pleiotrophin is a growth factor that induces carcinogenesis. Despite the fact that many published reports focused on the role of pleiotrophin and its receptors, receptor protein tyrosine phosphatase (RPTP β/ζ), and syndecan-3 during tumor development, no information is available regarding their function in tumor metastasis. To investigate the mechanism through which pleiotrophin regulates tumor metastasis, we used two different prostate carcinoma cell lines, DU145 and PC3, in which the expression of RPTP β/ζ or syndecan-3 was down-regulated by the RNAi technology. The loss of RPTP β/ζ expression initiated epithelial-to-mesenchymal transition (EMT) and increased the ability of the cells to migrate and invade. Importantly, the loss of RPTP β/ζ expression increased metastasis in nude mice in an experimental metastasis assay. We also demonstrate that RPTP β/ζ counterbalanced the pleiotrophin-mediated syndecan-3 pathway. While the inhibition of syndecan-3 expression inhibited the pleiotrophin-mediated cell migration and attachment through the Src and Fak pathway, the inhibition of RPTP β/ζ expression increased pleiotrophin-mediated migration and attachment through an interaction with Src and the subsequent activation of a signal transduction pathway involving Fak, Pten, and Erk1/2. Taken together, these results suggest that the loss of RPTP β/ζ may contribute to the metastasis of prostate cancer cells by inducing EMT and promoting pleiotrophin activity through the syndecan-3 pathway.

Pleiotrophin (PTN)2 (1), also known as heparin-binding growth-associated molecule (HB-GAM) (2), heparin-binding growth factor-8 (HBGF-8) (3), and heparin affin regulatory peptide (HARP) (4) is a developmentally regulated growth factor that shows limited expression in adult tissues but is markedly up-regulated in various primary human tumors such as small cell lung cancer (5), pancreatic cancer (6), melanoma (7), glioblastoma (8–10), and prostate cancer (11). Concomitantly, pleiotrophin is a proto-oncogene that functions as a mitogenic, anti-apoptotic, and tumor-transforming factor (4, 12–18). Furthermore, pleiotrophin stimulates epithelial-to-mesenchymal transition (EMT) (19), extensive remodeling of the malignant cell microenvironment (20), tumor angiogenesis (21, 22), and metastasis (7).

The pleiotropic functions of this growth factor are regulated primarily through the autocrine/paracrine effects of receptor-activated signaling. Receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ, PTPRZ1) (23, 24) and syndecan-3 (N-syndecan, SDC3) (25) have been characterized as pleiotrophin transmembrane receptors. Pleiotrophin is the first natural ligand discovered for any of the transmembrane tyrosine phosphatases and signals through the dimerization and inactivation of RPTP β/ζ (24). Whereas most PTPs are expressed in the peripheral tissues and CNS, RPTP β/ζ expression is primarily detected in the CNS (26). RPTP β/ζ is highly expressed in migrating neurons in the developing brain (27). Pleiotrophin stimulates the haptotactic migration of glioma cells, and the overexpression of pleiotrophin/RPTP β/ζ in human astrocytic tumor cells might create an autocrine loop that enhances cell migration (8, 9). Similar to RPTP β/ζ, syndecan-3 is abundantly expressed in the nervous system, particularly in the migrating neurons of the olfactory placode, providing indirect evidence that syndecan-3 mediates neural migration (30).

Despite the fact that there are many published reports describing the role of pleiotrophin and its receptors during tumor development, no information is available regarding their function during tumor metastasis. Furthermore, because RPTP β/ζ and syndecan-3 have not been closely studied together, it remains unclear whether their signaling cascades converge or act independently to promote the functional
response of pleiotrophin. In the present study, using RNAi technology, we stably transformed prostate carcinoma cells to down-regulate RPTPβ/ζ or syndecan-3 expression and investigated the in vivo and in vitro molecular mechanism through which pleiotrophin regulates tumor metastasis.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**—Cell culture reagents were from BiochromKG (Seromed, Germany). All other reagents were purchased from Sigma-Aldrich. Polyclonal antibodies against pSRC-kinase (Ytr-416), pFAK (Ytr-925), pPTEN (Ser-380), pAKT (Ser-473), pERK1/2 (Thr-202/Tyr-204), integrin-α5, -αv, -β1, -β3, E-cadherin, N-cadherin, and β-catenin, as well as monoclonal antibodies against SRC-kinase (36D10) and Snail (C1503), were purchased from Cell Signaling Technology. Polyclonal antibodies against HSC70 and Twist, and monoclonal antibodies against Slug were purchased from Santa Cruz Biotechnology, Inc. Monoclonal anti-RPTPβ/ζ antibodies and monoclonal antibodies against integrin-α5, -β1, and -β3 were from RD Systems and BD Transduction Laboratories (San Diego, CA). Polyclonal anti-syndecan-3 antibodies and monoclonal anti-Src were from Abcam and Upstate. Finally, Phalloidin-FITC was from Sigma-Aldrich.

**Purification of Human Recombinant Pleiotrophin**—Human recombinant pleiotrophin was produced in prokaryotic expression systems, as previously described (31). Pleiotrophin was further purified to remove LPS, and its biological activity was verified by the in vitro neurite outgrowth test.

**Attachment Assay**—24-well culture plates were coated with 10 μg/ml fibronectin, laminin, or collagen I for 1 h at 37 °C. Wells were then incubated with a 0.5% solution of bovine serum albumin (BSA) for 1 h at 37 °C to further block any specific adsorption of protein. 60,000 suspended cells in RPMI 1640 medium supplemented with 2.5% FBS were then seeded. After a 10-min incubation period, unattached cells were removed by shaking the plates at 2,000 rpm for 10 s and by three washes with PBS. Attached cells were fixed with 4% paraformaldehyde and stained with crystal violet.

**Crystal Violet Assay**—Adherent cells were fixed with methanol and stained with 0.5% crystal violet in 20% methanol for 20 min. After gentle rinsing with water, the retained dye was extracted with 30% acetic acid and the absorbance measured at 590 nm.

**Transwell Assay**—Migration assays were carried out in Boyden chambers using filters (8-μm pore size, Costar, Avon, France) coated with fibronectin, laminin, or collagen I (7.5 μg/cm2) for 1 h at 37 °C. Filters were washed, blocked with 0.5% BSA for 1 h at 37 °C, and dried. Assay medium (RPMI 1640 medium supplemented with 2.5% FBS, and 0.5% BSA with or without the chemo attractant) was added to the lower compartment, and 10^5 cells were added into the insert. After incubation for 30 min at 37 °C, filters were fixed. Non-migrated cells were scraped off the upper side of the filters and the filters were stained with crystal violet. The number of migrated cells was quantified by counting the entire surface area of the filter.

**Tail-vein Injections of Human Prostate Cancer Cells**—All in vivo experiments were carried out with ethical committee approval and under the condition established by the European Community. 1 × 10^6 DU145, DU145-NC2, or DU145-RM6 cells were suspended in 0.2 ml of PBS and injected into the lateral tail vein of athymic nude mice (Janvier), using a 27-gauge needle. The mice were sacrificed 10 weeks after injection, and the lungs were perfused with 1.5 ml of 15% India ink dye in 3.7% formalin. Lungs were then removed, rinsed in water for 15 s, and bleached in Fekete’s solution. Lung surfaces were photographed and scored.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**—Total RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The integrity of the isolated RNA was examined by electrophoresis on a 1% agarose gel containing 0.5 mg/ml ethidium bromide. Specific primers were as follows: hRPTPβ/ζ, 5'-TTCTGTGCTGCTGCAACCCCTTA-3' and 5'-AGGAAAGGAAAGAACATGCTCA-3'; hsyndecan-3, 5'-AGATGAGCAGTGTTGACCAAGAA-3' and 5'-CAAATCGAGAGCTCTCGAC-3'; hGAPDH, 5'-CCACCATGGCATAATCATGGG-3' and 5'-TCTAGACCGCAGTCAAGTCCACC-3'. The RT-PCR reactions were carried out in a single step with 250 ng of total RNA, using the Qiagen RT-PCR system. RT-PCR products were subjected to electrophoresis on 1% agarose gel containing 0.5 mg/ml ethidium bromide, digitally photographed, and quantified using image analysis software (Scion Image PC, Scion Corporation, Frederick, MD).

**siRNA Transfection**—RNA oligonucleotide primers and the siPORT NeoFX Transfection Agent were obtained from Ambion Inc. The following sequences were used: siRNA1 RPTPβ/ζ sense, 5'-UAUUGGUAUCCUAUGCUU-3'; siRNA2 RPTPβ/ζ antisense, 5'-AUCGCUUAGGAAUGCGC-3'; siRNA2 RPTPβ/ζ sense 5'-GCGACCAACUGAU-UUGUCCGA-3'; siRNA2 RPTPβ/ζ antisense, 5'-UGCAGAAUCAGUUGGUGGC-3'; siRNA1 syndecan-3 sense, 5'-UUCGUAAUGAAGAAATTT-3'; siRNA1 syndecan-3 antisense, 5'-UUCUUCAUACGAUAGUAG-3'; siRNA2 syndecan-3 sense, 5'-ACUGUAGAACCUCUCUCUGU-3'; siRNA2 syndecan-3 antisense, 5'-CGAGAGGAGUAGGU CC-3'. Annealing of the primers and transfection was carried out according to Ambion’s instructions. Briefly, siPORT NeoFX and siRNA were mixed at a final ratio of 1:10 in OPTI-MEM media. The transfection complexes were then overlaid onto 6-well plate cultures grown in RPMI-1640 supplemented with 10% FBS. Transfection efficiency was evaluated using Silencer FAM-Labeled GAPDH siRNA (Ambion).

**shRNA Transfection**—The pSilencer 4.1-CMV expression vector and the siPORT XP-1 Transfection Agent were obtained from Ambion Inc. Based on the siRNA sequence, shRNA was designed, ligated into the pSilencer 4.1-CMV expression vector, and transfected into cells according to Ambion’s instructions. Briefly, siPORT XP-1 and shRNA were mixed at a final ratio of 1:6 in OPTI-MEM media. The transfection complexes were then overlaid onto 24-well plate cultures grown in RPMI-1640 media, using the siPORT XP-1 Transfection Agent. Transfection efficiency was evaluated using Silencer FAM-Labeled GAPDH siRNA (Ambion).
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1640 supplemented with 10% FBS. After 1 month of selection with 300 μg/ml G418, clones were screened for down-regulation of RPTPβ/ξ expression. Double-stranded negative control shRNA from Ambion was also used.

**Immunoprecipitation**—Media from cell cultures grown in 60-mm plastic dishes were aspirated, cells were washed twice with ice-cold PBS, and lysed in 1 ml of buffer containing 50 mM HEPES pH 7.0, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Cells were harvested, sonicated for 4 min on ice, and centrifuged at 20,000 × g for 10 min at 4 °C. Approximately 600 μg of the supernatant was then incubated with 30 μl of protein A-Sepharose bead suspension for 60 min at room temperature. Beads were collected by centrifugation and the supernatants of the supernatant were then incubated with 30 μl of protein A-Sepharose bead suspension for 60 min at room temperature.

**RESULTS**

**Knockdown Induces an EMT-like Phenotype**—As shown in Fig. 1B, the cell lines silenced for RPTPβ/ξ migrated more robustly (100% higher) than the parental lines or the cells transfected with control vector, DU145-NC, DU145-NC2, PC3-NC, and PC3-NC4. Furthermore, RPTPβ/ξ attenuation also increased the invasive capacity of the cells (130% increase) (Fig. 1C). To determine the effect of RPTPβ/ξ on the metastatic dissemination of human prostate cancer cells, DU145, DU145-NC2, or DU145-RM6 cells were injected into nude mice through the tail veins. The lungs were harvested after 10 weeks and scored for metastasis. As shown in Fig. 1D, the mice that were injected with DU145-RM6 cells developed lung metastasis, but not those injected with DU145 or DU145-NC2 cells. This is the first demonstration that RPTPβ/ξ knockdown increases the intrinsic ability of prostate cancer cells to metastasize *in vivo*. Taken together, these results suggest that RPTPβ/ξ may act as a tumor suppressor for prostate cancer metastasis.
...sion increased the fibroblastic morphology of the cells as evidence by their elongated shape and the formation of multiple membrane protrusions. Phalloidin staining of RPTPβ/ζ knockdown cells revealed increased stress fiber formation and a concomitant decrease in the number of cortical actin fibers, marker of lamellipodia formation. This phenotype, in combination with the observed increased cell motility and invasion (Fig. 1), prompted us to examine whether RPTPβ/ζ knockdown is associated with EMT.

The expression profile of adhesion molecules, such as integrin-α5, -αv, -β1, and -β3 was investigated by Western blot and FACS analysis. The down-regulation of RPTPβ/ζ induced the expression of integrin-α5, -αv, and -β3 (Fig. 2, B and C). Furthermore, there was a shift in cadherin expression from the E- to the N-cadherin form, known to characterize the EMT-like phenotype and to promote cell motility (33). As shown in the immunoblots and immunofluorescence staining in Fig. 2, B and D, E-cadherin expression was clearly decreased in DU145-RM6 or PC3-RM1 cells compared with wild-type cells, while that of N-cadherin was increased. Fig. 2, B and D also shows that DU145-RM6 and PC3-RM1 cells expressed higher levels of Twist and Slug/Snail transcription factors, known to down-regulate E-cadherin (34).

The down-regulation of RPTPβ/ζ also induced the translocation of β-catenin form the cell surface to the nucleus (Fig. 2D). Hence, down-regulation of RPTPβ/ζ induces an EMT-like phenotype.

The Effect of RPTPβ/ζ or Syndecan-3 Down-regulation on Pleiotrophin-mediated Migration and Attachment—We next examined the response of the prostate cancer cell lines silenced for RPTPβ/ζ to its ligand pleiotrophin. For that, we tested the effect of exogenously added pleiotrophin on cell migration using both Boyden chamber and wound closer assays. In the Boyden chamber assay, an equal number of cells from each cell line were plated in inserts coated with fibronectin and allowed to migrate toward increasing concentrations of recombinant pleiotrophin ranging from 5 to 100 ng/ml. The cells that
migrated across the 8-μm porous membrane were fixed, stained, and counted. As expected, pleiotrophin increased migration (40% increase) in the wild-type DU145 and PC3 cells. However, RPTPβ/ζ gene silencing caused a greater increase (2-fold) in these cells and when the RPTPβ/ζ silenced cells, DU145-RM, DU145-RM6, PC3-RM, and PC3-RM1, were treated with pleiotrophin, a further increase in cell migration was observed, which was pleiotrophin concentration dependent (data not shown), with a maximal effect (267% increase) obtained at a concentration of 50 ng/ml (Fig. 3A). These results suggest that the binding of pleiotrophin to RPTPβ/ζ inhibits pleiotrophin-mediated migration and that the observed induction in the RPTPβ/ζ silenced cells may therefore be mediated through syndecan-3, the pleiotrophin other receptor. To test this hypothesis, syndecan-3 expression was transiently down-regulated in both cell lines, using two different siRNA sequences (Fig. 3B). As shown in Fig. 3A, the syndecan-3 silenced cells, DU145-SM1, DU145-SM2, PC3-SM1, and PC3-SM2, migrated less effectively than the parental lines. Moreover, treatment with pleiotrophin further decreased DU145-SM1, DU145-SM2, PC3-SM1, and PC3-SM2 migration in a concentration-dependent manner (data not shown), with a maximal effect (70% decrease) at 50 ng/ml. All migration experiments were also performed on laminin or collagen I as coating substrates and gave similar results (data not shown). The effect of pleiotrophin on the migration of DU145-NC, DU145-NC2, DU145-RM6, PC3, PC3-NC4, and PC3-siNC cells (cells transfected with control vector) was similar to the effect observed on DU145 or PC3 cells. The wound-closure assays confirmed the inhibitory effect of RPTPβ/ζ on pleiotrophin-mediated motility (Fig. 3C). These results demonstrate that the pleiotrophin/RPTPβ/ζ interaction inhibit cell migration whereas the pleiotrophin/syndecan-3 interaction induces cell migration.

Because cell migration depends on cell attachment, we examined the effect of pleiotrophin on the attachment of
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FIGURE 3. Pleiotrophin-mediated migration and attachment is inhibited by RPTPβ/ζ and induced by syndecan-3. A, effect of pleiotrophin on the migration of DU145, DU145-NC, DU145-NC2, DU145-RM, DU145-RM6, DU145-siNC, DU145-SM1, DU145-SM2, PC3, PC3-NC, PC3-NC4, PC3-RM, PC3-RM1, PC3-siNC, PC3-SM1, and PC3-SM2 cells on Transwell filters (8-μm pores) coated with fibronectin. B, siRNA-mediated syndecan-3 knockdown in DU145 and PC3 cells. Western blot analysis of syndecan-3, RPTPβ/ζ, and pleiotrophin. DU145-siNC and PC3-siNC cells transfected with negative control siRNA, DU145-SM1, DU145-SM2, PC3-SM1, and PC3-SM2 cells transfected with two different syndecan-3 siRNA sequences. HSC70 was used as a loading control. C, wound closure assay in DU145, DU145-NC2, DU145-RM6, PC3, PC3-NC4, and PC3-RM1 cells. The images were captured at 0 and 30 h after wounding in growth medium with or without pleiotrophin. D, effect of pleiotrophin on the attachment of DU145, DU145-NC, DU145-NC2, DU145-RM, DU145-RM6, DU145-siNC, DU145-SM1, DU145-SM2, PC3, PC3-NC, PC3-NC4, PC3-RM, PC3-RM1, PC3-siNC, PC3-SM1, and PC3-SM2 cells, on fibronectin coated plates. The results are given as the mean values ± S.D. of at least three independent experiments.

RPTPβ/ζ or syndecan-3 knockdown cells. An equal number of cells were pre-incubated with increasing concentrations of pleiotrophin before seeding into culture wells coated with fibronectin and allowed to attach for 10 min. As shown in Fig. 3D, DU145-RM, DU145-RM6, PC3-RM, and PC3-RM1 cells were attached more efficiently than the parental control cells and pleiotrophin further increased DU145-RM, DU145-RM6, PC3-RM, and PC3-RM1 cell attachment, with a maximal effect (240% increase) obtained at a concentration of 50 ng/ml. However, the syndecan-3 knockdown DU145-SM1, DU145-SM2, PC3-SM1, and PC3-SM2 cells attached less effectively than the wild-type cells and their number was further decreased after pleiotrophin treatment in a concentration-dependent manner (50% inhibition at 50 ng/ml). These results indicate that pleiotrophin/RPTPβ/ζ interaction inhibits cell attachment, while in contrast, the pleiotrophin/syndecan-3 interaction induces cell attachment.

Opposing Effect of Pleiotrophin/RPTPβ/ζ and Pleiotrophin/Syndecan-3 Pathways on Src and Fak Activation—Pleiotrophin signals through the dimerization and inactivation of RPTPβ/ζ, and the loss of phosphatase activity results in increased tyrosine phosphorylation levels of different substrates, including β-catenin (24), β-adducin (36, 37), Fyn (38), and GIT1/Cat1 (39). However, our results show that the pleiotrophin-mediated RPTPβ/ζ inactivation is associated with an inhibition of cell migration and attachment. To look into the mechanism involved in the inhibition of migration we searched for signal transduction molecules whose activation depends on dephosphorylation events. Src appeared as a potential candidate as its activation is strictly regulated by and depends on the dephosphorylation of Tyr-527 in its C-terminal tail, which is a prerequisite for the autophosphorylation of Tyr-416 in the activation loop and the subsequent activation of the kinase (40). After demonstrating by immunoprecipitation experiments that Src interacted with RPTPβ/ζ (Fig. 4A), we examined the effect of pleiotrophin on Src phosphorylation in our knockdown DU145 cells. DU145, DU145-NC2, DU145-RM6, and DU145-SM1 cells were serum-starved for 4 h and incubated with increasing concentrations of pleiotrophin for 3 to 45 min. Src activation was indirectly assessed using Western blot analysis of phosphorylated Src at site Tyr-416. Levels of HSC70 were used as loading control. Within 3 min, pleiotrophin promoted a rapid increase in Src phosphorylation in a concentration-dependent manner, with a maximal effect (150% induction relative to control) at 50 ng/ml (Fig. 4B). This was followed by a return to near basal levels at 10 to 30 min; a second, weaker increase in Src phosphorylation occurred 45 min after pleiotrophin treatment (data not shown). However, the effect of pleiotrophin on the Src-mediated phosphorylation in DU145-RM6 cells (RPTPβ/ζ knockdown) was markedly stronger, with a maximal induction of 350% occurring in the presence of 50 ng/ml pleiotrophin. These results indicate that the binding of pleiotrophin to RPTPβ/ζ inhibits Src activation in wild-type cells and that the observed induction in the RPTPβ/ζ knockdown cells may therefore be mediated through syndecan-3. As shown in Fig. 4, A and B, syndecan-3 interacted with Src and pleiotrophin inhibited Src phosphorylation in the syndecan-3 knockdown DU145-SM1 cells in a concentration-dependent manner, with a maximal effect (60% decrease) at 50 ng/ml. Furthermore, we observed an increased interaction between Src and syndecan-3 in the RPTPβ/ζ silenced DU145-RM6 cells, an increased interaction between Src and RPTPβ/ζ cells in the syndecan-3 silenced DU145-SM1 cells, but no interaction between the RPTPβ/ζ and the syndecan-3, indicating that there are two distinct signal transduction pathways (Fig. 4A). Taken together, our results suggest that the binding of pleiotrophin to RPTPβ/ζ inactivates Src and counterbalances the pleiotrophin/syndecan-3-mediated induction of Src phosphorylation.
To study the functional impact of Src activation on pleiotrophin-mediated biological actions, we used the pharmacological inhibitor PP1 to specifically block the Src pathway. As shown in FIG. 4C, PP1 abrogated the effect of pleiotrophin on cell migration and attachment. Hence, the results suggest that Src is a key regulator of pleiotrophin-mediated migration and attachment.

We next examined the effect of pleiotrophin on other molecules known to interact with Src. We found that Fak phosphorylation was increased in a concentration-dependent manner 3 min after the incubation of DU145 cells with pleiotrophin with maximal effect (80% increase) at 50 ng/ml pleiotrophin (Fig. 4C). This activation was followed by a return to basal levels at 10 to 30 min after pleiotrophin treatment (data not shown). However, Fak phosphorylation by pleiotrophin was much greater in DU145-RM6 cells (150% increase) while it was inhibited in DU145-SM1 cells (70% decrease) (Fig. 4C). Hence, similarly to Src, the pleiotrophin/RPTPβ/ζ interaction inactivates Fak and counterbalances the pleiotrophin/syndecan-3-mediated Fak activation.

RPTPβ/ζ, but Not Syndecan-3, Induces Pten and Erk1/2 Dephosphorylation after Pleiotrophin Treatment—We further tested whether pleiotrophin affects Pten or Erk1/2 activation. We found that Pten phosphorylation was decreased 3 min after the incubation of DU145 cells with pleiotrophin with maximal effect (60% inhibition relative to control) at 50 ng/ml pleiotrophin (Fig. 4C). This activation was followed by a return to basal levels at 10 to 30 min after pleiotrophin treatment (data not shown). Contrary to the effect on DU145 cells, pleiotrophin had no effect on Pten phosphorylation in DU145-RM6 cells (Fig. 5A). Furthermore, the pleiotrophin effect on Pten phosphorylation in DU145-SM1 cells was similar to the observed effect on wild-type cells (65% inhibition relative to control at a concentration of 50 ng/ml) (Fig. 5A). Similar to Pten activation, the extracellular signal-regulated kinases 1 and 2 were also inhibited by pleiotrophin in DU145-SM1 cells (Fig. 5B).
Our results show that RPTPβ/ζ was a negative regulator of EMT, where carcinoma cells lose polarity, cell–cell contacts and other epithelial characteristics, switching to a motile mesenchymal phenotype (45, 46). Common EMT features include a loss of E-cadherin, elevated N-cadherin, integrins, Twist, Slug, and Snail expression, increased nuclear β-catenin, and gain of fibroblastoid morphology. Reduced E-cadherin expression has been observed in high-grade prostate cancers and is associated with poor prognosis (47, 48). Prostatic epithelial cells undergo EMT in response to abnormal receptor activation, such as platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), transforming growth factor-β receptor (TGF-βR), and insulin-like growth factor-1 receptor (IGF-1R) (46). Previous study has shown that in glioma cells, pleiotrophin also initiates EMT (19). In the present work however, we show that the down-regulation of the expression of its receptor RPTPβ/ζ induced EMT and increased the ability of human prostate cancer cells to migrate and invade in vitro and to metastasize in vivo. These results are consistent with recent data available in the MSKCC database that indicate that RPTPβ/ζ expression is reduced up to 50% in primary prostate cancer, whereas in the case of metastatic androgen-dependent or androgen-independent prostate tumors, this percentage reaches 67 and 35%, respectively. However, it seems that the effect of RPTPβ/ζ on cell motility is not the same in all types of cancers. Previous studies in neuroblastoma have shown that down-regulation of RPTPβ/ζ reduces haptotactic activity (50). In gliomastoma the expression of RPTPβ/ζ is increased compared with normal brain tissues (8) and in human astrocytic tumor cells, the up-regulated expression of RPTPβ/ζ and pleiotrophin creates an autocrine loop that is important for cell migration (9). However, pleiotrophin and RPTPβ/ζ expression were found decreased in human colorectal cancers (51).

Our results also show that RPTPβ/ζ decreased pleiotrophin-mediated migration and attachment through an interaction with Src, triggering a signal transduction pathway that inactivates Fak, and Erk1/2 and activates Pten, molecules frequently deregulated in prostate cancer. Specifically, Pten, a tumor suppressing molecule, is the most frequently mutated gene involved in prostate cancer metastasis. Acquired mutations in the Pten gene are associated with advanced stage and poor prognosis (52). Pten, which is activated after dephosphorylation, dephosphorylates Fak, and reduces cell migration and focal adhesion formation (53). Furthermore, Pten negatively regulates the PI3K and MAPK pathway and its loss leads to constitutive activation of the Erk1/2, which regulates motility in prostate cancer cells (54). However, unlike RPTPβ/ζ, the interaction of pleiotrophin with its second receptor, syndecan-3, induces prostate cancer cell migration, suggesting that the effect of pleiotrophin on cell migration is the overall result of the opposing actions of its two receptors, RPTPβ/ζ and syndecan-3. Although pleiotrophin is known as a growth factor with anti-apoptotic and angiogenic activities, other studies, like ours, already suggested that pleiotrophin can also function as a negative regulator of biological responses. For example, it was shown to promote the apoptotic response of cardiomyocytes through the inhibition of Akt signaling (55) and to inhibit angiogenesis by interfering with VEGF165 (56). Moreover, it

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FIGURE 6. The Src/Fak pathway is related to the EMT phenotype. Western blot analysis of phosphorylated Src (A) and Fak (B) in DU145, DU145-NC2, DU145-RM6, PC3, PC3-NC4, and PC3-RM1 cells. The blots were stripped and reprobed for HSC70 as a loading control. The results are given as the mean values ± S.D. of at least three independent experiments.
has been shown that pleiotrophin negatively regulates adipogenesis (35). In addition, several publications have reported contradictory results concerning the activities of pleiotrophin. While only the recombinant polypeptide produced in a mammalian system stimulated the proliferation of fibroblast, endothelial, and epithelial cells (1, 29, 44), pleiotrophin produced in prokaryotic expression systems had a mitogenic effect on endothelial cells only when it was immobilized on the culture plate (31). Furthermore, eukaryotic-produced pleiotrophin in solution had no significant effect on the proliferation and chemotactic migration of glioma cell lines (8, 9, 28), but when presented as an immobilized substrate, it strongly stimulated them (9). The explanations provided for these contradictory results focused mainly on pleiotrophin folding, modifications and on the type of expression system used. A more recent study using glioma cell lines proposed the expression of two different forms of pleiotrophin, each displaying distinct biological actions; a full-length form which induced haptotactic migration, whereas the shorter, 15 kDa form, activated a mitogenic signal transduction pathway (49). In our study however, only purified fractions of full-length pleiotrophin, produced in prokaryotic expression systems were used. Our present study showing opposing effects of the two pleiotrophin receptors provides therefore a new reasoning for pleiotrophin conflicting effects, as they would possibly depend on their relative expression levels.

In summary, we suggest that RPTPβ/ζ plays a tumor suppressor-like role in prostate cancer metastasis. Fig. 7 schematically describes our views on how the expression of RPTPβ/ζ may influence tumor progression. During early stages of prostate tumor development, both RPTPβ/ζ and syndecan-3 would be expressed and RPTPβ/ζ would counterbalance the pleiotrophin-mediated syndecan-3 pathway, reducing migration. However, inactivation of RPTPβ/ζ or loss of its expression which may occur during tumor progression would initiate EMT. In these cells, pleiotrophin would be left to interact with syndecan-3, increasing cell migration, invasion and metastasis. This work provides the first evidence of the molecular mechanism through which pleiotrophin and its receptors RPTPβ/ζ and syndecan-3 regulates prostate cancer metastasis. These results may contribute to more effective therapeutic strategies for the treatment of prostate cancer and warrant further study.

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