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Cobalt Chloride-Induced Downregulation of Puromycin-Sensitive Aminopeptidase Suppresses the Migration and Invasion of PC-3 Cells

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Cobalt chloride ($CoCl_2$) treatment of cells *in vitro* has been shown to induce cellular changes that are similar to those seen following hypoxia. To identify genes that are differentially expressed in response to treatment with $CoCl_2$, we compared the mRNA expression profiles of PC-3 cells that were treated with $CoCl_2$ with those of untreated PC-3 cells, using specific arbitrary primers and two anchored oligo(dT) primers provided in the ACP-based GeneFishing kits. The results of this study demonstrated that the puromycin-sensitive aminopeptidase (PSA) gene was downregulated in PC-3 cells that were treated with $CoCl_2$. This downregulation of PSA expression, in turn, suppressed the proliferation, migration, and invasion of PC-3 cells, as well as the secretion and expression of matrix metalloproteinase-9 (MMP-9).

Keywords: Cobalt chloride, PSA, migration, MMPs, angiogenesis

Angiogenesis, the growth and proliferation of new blood vessels, is a crucial process in the physiologic and pathologic responses inherent to a variety of disease conditions, including wound repair, tumor growth, and rheumatoid arthritis [10, 11, 17]. In addition, angiogenesis is an essential event involved in the progression of tumors and metastasis [2]. Furthermore, degradation of the extracellular matrix (ECM) surrounding primary tumors and metastasis is critical for invasion and metastasis of epithelial tumor cells [6]. The zinc-dependent endopeptidases, MMPs, can degrade ECM and are involved in cancer invasion, metastasis, and angiogenesis [9].

Hypoxia is a reduction in the level of tissue oxygen tension that occurs during acute and chronic vascular disease and pulmonary disease. Hypoxic conditions in tumors induce the release of cytokines that promote vascularization, thereby enhancing tumor growth and metastasis [4]. In addition, normal tissue function requires an adequate supply of oxygen through the blood vessels. Accordingly, focal areas of low oxygen tension (<2.0%) are inherent to the biological processes of embryogenesis and wound repair [12, 24]. $CoCl_2$ treatment of cells *in vitro* has previously been shown to induce cellular changes that are similar to those seen following hypoxia [5]. In addition, $CoCl_2$ is known to activate hypoxia inducible factor-1 (HIF-1) transcription factor, which is a key regulator of the angiogenic response in hypoxia.

Aminopeptidases are metalloproteinases that remove amino acids from the N-termini of proteins and function during posttranslational modification. It has been reported that some aminopeptidases, including methionine aminopeptidase type 2 (MetAP2) [13], aminopeptidase N (APN)/CD13 [3], aminopeptidase A (APA) [20], and adipocyte-derived leucine aminopeptidase (A-LAP), play an important role in angiogenesis [23, 27]. In addition, puromycin-insensitive leucyl-specific aminopeptidase (PILSAP) is expressed in endothelial cells (ECs) at the site of postnatal angiogenesis, and specific elimination of PILSAP expression abrogates the proliferation and migration of vascular endothelial growth factor (VEGF)-induced ECs *in vitro*, as well as VEGF- or basic fibroblast growth factor (bFGF)-induced angiogenesis *in vivo* [21]. Although PSA was initially purified as an enkephalin-degrading aminopeptidase, it has been proposed that it functions in a number of processes including the metabolism of neuropeptides [15], regulation of the cell cycle [7], processing of antigenic peptides for presentation on MHC class I molecules, and hydrolysis of proteasomal products to amino acids [22, 25].

Cells respond to hypoxia by selective gene induction and downregulation [19]. Therefore, we compared the mRNA expression profiles of PC-3 cells that were treated with $CoCl_2$ with those of a non-treated group to identify genes that were differentially expressed in response to $CoCl_2$. In this study, we demonstrated that the PSA gene was downregulated in PC-3 cells that were treated with

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$CoCl_2$ and that PSA significantly inhibits the proliferation, migration, and invasion of PC-3 cells.

MATERIALS AND METHODS

Materials

Recombinant human MMP-2, MMP-9, TIMP-1, and TIMP-2 enzyme immunoassay kits were purchased from R&D Systems (Minneapolis, MN, U.S.A.). MMP-2, MMP-9, HIF-1α, and HIF-2α antibodies were purchased from Calbiochem (La Jolla, CA, U.S.A.). The media and sera used in this study were acquired from Life Technology, Inc. (Gaithersburg, MD, U.S.A.). The GeneFishing DEG101 system was obtained from SeeGene (Seoul, Korea). The remainder of the biochemical reagents used in this study, including gelatin, fibrinogen, Giemsa staining solution, antibiotics, antimycotics, and trypsin-EDTA, were purchased from Sigma (St. Louis, MO, U.S.A.), unless otherwise specified.

Cell Culture and Inhibition of PSA Expression by Small Interfering RNAs (siRNA)

Human prostate adenocarcinoma cell line PC-3 cells were acquired from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were maintained at 37°C under 5% CO_2 in RPMI-1640 that was supplemented with 10% (v/v) heat-inactivated FBS. For siRNA transfection, PC-3 cells (5×10^5) were washed in sterile PBS and then replenished in supplemented RPMI-1640 media for 24 h. The PC-3 cells were then transfected with 3 independent siRNA oligonucleotides (Bioneer, Seoul, Korea) that targeted the gene responsible for production of human PSA. The coding strands of the three pairs of siRNA oligonucleotide directed at the 5' end of the PSA mRNA were 5'-GCUCGAGCUGGAAUCAUUATT-3', 5'-TTUAAUGAUUCCAGCUCGAGC-3'; 5'-GCUGCUUGGAAAUUCAUAATT-3', 5'-TTUUAUGAAUUUCCAAGCAGC-3'; and 5'-GCUAUCAGUUGAGGGAUUUTT-3', 5'-TTAAAUCCCUCAACUGAUAGC-3'. In addition, the following scramble RNAs (Scr siRNA) was used as negative controls: 5'-CCUACGCCACCAAUUUCGUTT-3' and 5'-TTACGAAAUUGGUGGCGUAGG-3'. The transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's protocols. Knockdown of PSA was then assessed by Western blotting, 48 h after transfection.

Screening of Differentially Expressed Genes (DEGs)

To screen for DEGs in PC-3 cells that were treated with $CoCl_2$, we used ACP-based GeneFishing DEG kits according to the manufacturer's instructions. Briefly, first-strand cDNAs were synthesized with total RNA extracted from PC-3 cells using MMLV-RT (Promega, Madison, WI, U.S.A.) and dT-ACP1 primer (5'-CTGTGAATGCTGCGACTACGATIIIIIT$_{18}$-3'). Second-strand cDNA was then synthesized using primers specific for dT-ACP2 and arbitrary ACP in sequential one-cycle reactions that were as follows: 94°C for 1 min, 50°C for 3 min, and 72°C for 1 min. Following second-strand cDNA synthesis, second-stage PCR amplification was carried out by subjecting the samples to the following conditions: 40 cycles of 94°C for 40 s, 65°C for 40 s, and 72°C for 40 s, followed by a 5-min final extension at 72°C. The amplified cDNA fragments with differential band intensities were then re-amplified and extracted from the gel using the PCR Clean Up system (Promega, Madison, WI, U.S.A.), after which they were cloned using TOPO-TA cloning kits (Invitrogen, Frederick, MD, U.S.A.). The complete sequences were then analyzed by searching for similarities using the BLASTN search program at the National Center for Biotechnology Information.

RNA Extraction and RT-PCR

PC-3 cells were cultured in 10% FBS (v/v) RPMI-1640 to approximately 80% confluence. The cells were then incubated in RPMI-1640 supplemented with 5% FBS (v/v) for 20 h. Next, the medium was exchanged for serum-free medium that was amended with either control buffer or $CoCl_2$ (100 μM). The total RNA was then extracted with TRIzol reagent (MRC, Cincinnati, OH, U.S.A.) in accordance with the manufacturer's instructions. One μg of total RNA was then reverse-transcribed using ImProm-II reverse transcriptase (Promega, Madison, WI, U.S.A.). Next, a 3-μl aliquot of the reverse transcription reaction was used as the template for PCR amplification of the cDNA fragments using primers specific for PSA (sense primer, 5'-GTGAGGCAGGCGACTAATCAG-3'; antisense primer, 5'-CTGGGCACGGGTGTAATCAGC-3') and β-actin cDNA (sense primer, 5'-AAGGATTCCTATGTGGGCGAC-3'; antisense primer, 5'-GCTCGGTGAGGATCTTCATGA-3') as an internal control.

Proliferation Assay

Control PC-3 cells and siRNA-transfected cells were seeded in 96-well culture plates at a density of 1×10^4 cells per well. The cells were then incubated for 1 to 3 days before being applied to Cell Titer 96 aqueous cell proliferation assay kits (Promega, Madison, WI, U.S.A.). The absorbance of the solution was read at 490 nm using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Migration and Invasion Assay

Migration of PC-3 cells was evaluated using a QCM 24-well colorimetric cell migration assay kit (Chemicon, Temecula, CA, U.S.A.). PC-3 cells were plated at 1×10^5 cells/well in RPMI-1640 containing 10% FBS onto the upper compartment of the chamber. For invasion assays, 1×10^5 cells were seeded onto the upper compartment of Matrigel-coated chambers with a filter membrane containing 8-μm pores (Beckton Dickinson, Bedford, MA, U.S.A.). The filter was allowed to dry, after which it was stained and transferred to a clean well containing 200 μl of extraction buffer. The absorbance of the solution at 560 nm was then read using an Emax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Enzyme Immunoassay of MMPs and TIMPs

PC-3 cells were seeded onto 24-well plates at a density of 5×10^4 cells/cm^2 and then grown for 24 h in RPMI-1640 supplemented with 20% FBS. Next, the confluent PC-3 cells were incubated in serum-free and phenol-red-free RPMI-1640 for 12 h. The cells were then washed in fresh medium, after which a control buffer or the indicated reagents were applied for 12 h. The actual quantities of the MMPs and TIMPs were then determined *via* an enzyme immunoassay.

Gelatin Zymography

PC-3 cells were seeded in 24-well plates at a density of 5×10^4 cells/cm^2, and then grown for 24 h in RPMI-1640 supplemented with 20% FBS. The confluent PC-3 cells were incubated for 12 h in serum-

free and phenol-red-free RPMI-1640. The cells were then washed in fresh medium, after which a control buffer or the indicated reagents were applied for 12 h. Next, the samples were mixed with 5× sample buffer (4 M Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, and 0.1% bromophenol blue) and then subjected to 10% SDS-PAGE using gels that contained 0.1% gelatin. The gels were then incubated for 1 h in 2.5% Triton X-100, after which they were incubated for 24 h in enzyme buffer (0.05 M Tris-HCl, pH 7.5, 0.02 M NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35) at 37°C. The gels were then stained with 0.5% Coomassie Brilliant Blue 250 solution, and then destained by several washes with 30% methanol and 10% acetic acid.

Western Blot Analysis

The cells were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and 1 mM EGTA). The lysed cells were then centrifuged for 10 min at 14,000 ×g, after which the supernatants were transferred to clean tubes, and the proteins were quantitated *via* bicinchoninic acid protein assays (Pierce, Rockford, IL, U.S.A.). The protein samples (50 μg) were then resolved on 10% polyacrylamide SDS gels and then transferred to nitrocellulose Hybond-C Extra membranes. The membranes were then incubated with primary antibodies, after which they were incubated with horseradish peroxidase-conjugated secondary antibodies. They were then developed *via* enhanced chemiluminescence (ECL, Amersham International, Buckingham, England).

Data Analysis

Data were expressed as the means ± the standard deviation (SD). Statistical significance was assessed *via* one-way ANOVA, followed by the Student-Newman-Keuls test. Statistical significance was set at a P value of <0.05.

Results and Discussion

Expression of the PSA Gene was Downregulated in Response to Treatment with $CoCl_2$

When tumors encounter low oxygen tension, they adapt by regulating the expression of genes associated with anaerobic cell metabolism, cell survival, angiogenesis, metastasis, and invasion [8]. Hypoxia is generally considered to represent a fundamental stimulus for angiogenesis. Although most angiogenic factors are upregulated by hypoxia, a strong angiogenic factor, hepatocyte growth factor (HGF), is downregulated [26]. To identify genes that are differentially expressed in response to treatment with $CoCl_2$, we compared the mRNA expression profiles of PC-3 cells that were treated with $CoCl_2$ to those of a control group. Using specific arbitrary primers and two anchored oligo (dT) primers provided by the ACP-based GeneFishing PCR kits, several cDNA fragments that showed differential expression in response to treatment with $CoCl_2$ were identified. Specifically, the expression of glyceraldehyde-3-phosphate dehydrogenase was upregulated, whereas the expression of PSA (Fig. 1A), chaperonin containing TCP1 subunit 5, and ribosomal protein S23 were downregulated.

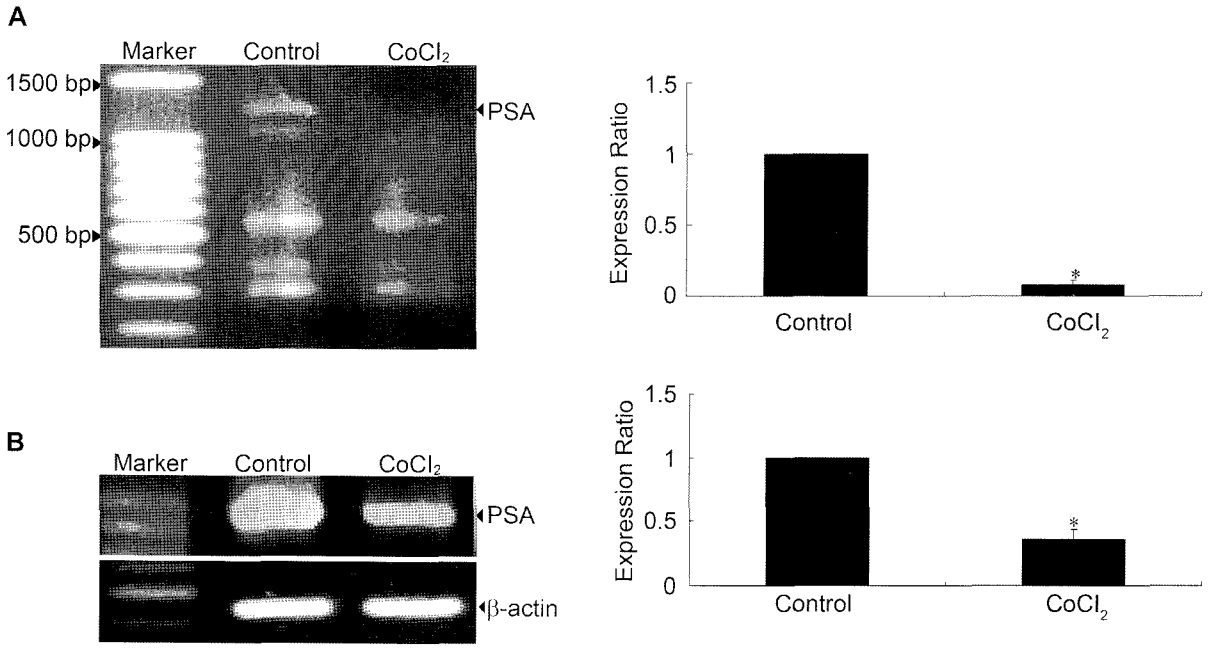

Fig. 1. The effect of $CoCl_2$ on the expression of the PSA gene.
Cells were incubated in 5% FBS (v/v) RPMI-1640 for 20 h. Next, the medium was changed to serum-free RPMI-1640 that was amended with a control buffer (Control) or $CoCl_2$ (100 μM). **A**. The arrow indicates a partial cDNA fragment of the PSA gene. The following sequences of ACP-based GeneFishing PCR primers that were specific for the PSA gene were used: dT-ACP2, 5'-CTGTGAATGCTGCGACTACGAT$_{15}$-3', and ACP7, 5'-GTCTACCAGGCATTCGCT-TCATCTGCGGATCG-3'. **B**. Confirmation by semiquantitative RT-PCR of the differential mRNA expression patterns of PSA that were identified by ACP RT-PCR. β-actin was utilized as a normalization control.

PSA Regulates the Proliferation, Migration, and Invasion of PC-3 Cells

Aminopeptidase is required as the third protease system following plasminogen activator (PA) and MMP, which are both related to angiogenesis. The roles of PA and MMP in angiogenesis are specific to the degradation of extracellular matrices. However, the role that aminopeptidase plays in angiogenesis is not clear. Proliferation and migration are principal properties involved in angiogenesis; therefore, to determine if PSA is involved in angiogenesis, we examined the proliferation, migration, and invasion of PC-3 cells after siRNA knockdown of PSA. The PSA protein was almost completely attenuated by the PSA siRNA (Fig. 2A). However, transfection with the control siRNA (Scr siRNA) had no effect on PSA levels (Fig. 2A). Because $CoCl_2$ downregulated the PSA expression (Fig. 1), we evaluated the effects of $CoCl_2$ on PC-3 cell proliferation. As shown in Fig. 2B, $CoCl_2$ inhibited the proliferation of PC-3 cells. In addition, treatment with puromycin, a PSA inhibitor, inhibited the proliferation of PC-3 cells, and this effect was enhanced by combined treatment of cells with $CoCl_2$ (100 μM) and puromycin (10 μM) (Fig. 2B). To determine if the loss of PSA has an effect on the ability of PC-3 cells to proliferate, we analyzed the effect of PSA siRNA expression on PC-3 cell proliferation. The proliferation of PC-3 cells that were transfected with PSA siRNA was significantly blocked by 62% at day 3 (Fig. 2B). In addition, the number of migrating cells was blocked by 37% and 30% in response to treatment with $CoCl_2$ (100 μM) and puromycin (10 μM), respectively. Combined treatment with $CoCl_2$ (100 μM) and puromycin (10 μM) produced an enhanced antimigrative effect (Fig. 2C). In this study, we confirmed that $CoCl_2$ and knockdown of PSA both exerted antiproliferative and antimigrative effects on PC-3 cells that were involved in angiogenesis. The marked reduction of PC-3 cell migration by PSA siRNA supports the assumption that PSA plays a role in the cell invasiveness of tumors. PSA has been proposed to be a target molecule of cell invasion inhibitors [16, 18]. As shown in Fig. 2D, $CoCl_2$ and puromycin produced approximately 46% and 39% suppression of the invasion of PC-3 cells, respectively, when compared with the addition of a control buffer. Additionally, combined treatment with $CoCl_2$ (100 μM) and puromycin (10 μM) reduced the invasion of PC-3 cells by approximately 65% (Fig. 2D). The invasive activity was significantly blocked by 68% in PSA siRNA transfected PC-3 cells (Fig. 2D). The results of a recent report suggested that PILSAP plays an important role in angiogenesis owing to its involvement in migration, proliferation, and network formation [1]. An important finding of this study is that silencing PSA markedly decreased the proliferation, migration, and invasion of PC-3 cells *in vitro*, which suggests that PSA is involved in angiogenesis.

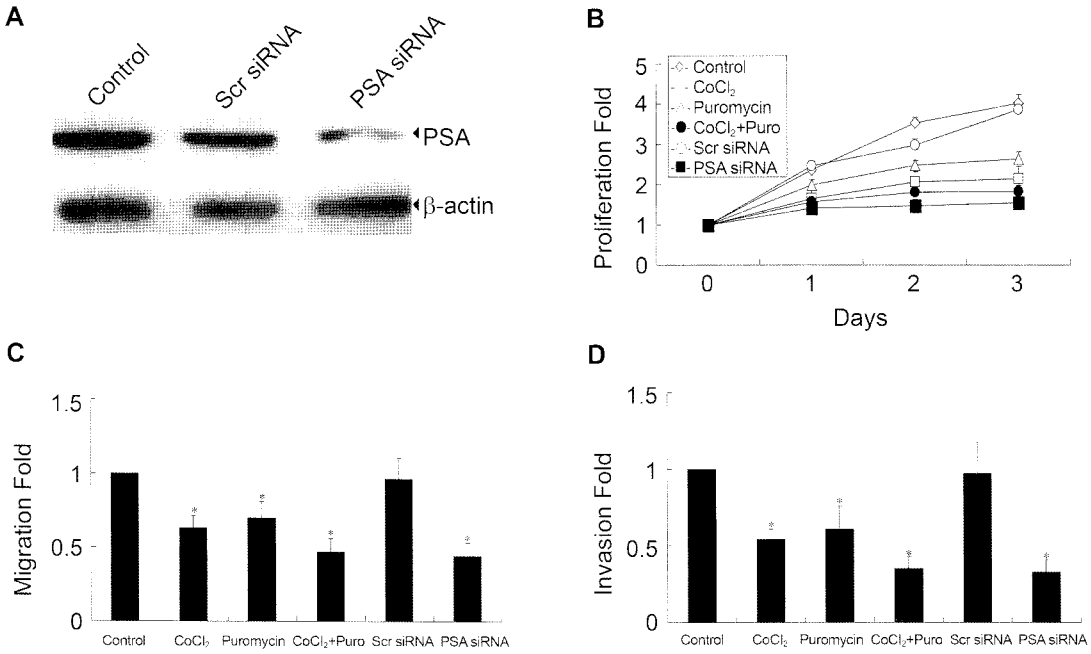

Fig. 2. The effects of $CoCl_2$, puromycin, and PSA siRNA on PC-3 cell proliferation, migration, and invasion.

Cells were incubated in 5% FBS (v/v) RPMI-1640 for 20 h. Next, the medium was changed to serum-free RPMI-1640 that was amended with control buffer (Control) or $CoCl_2$ (100 μM). **A**. Knockdown of PSA was assessed, at 48 h after transfection, by Western blotting. Scramble RNAs (Scr siRNA) were used as negative controls. Cell proliferation (**B**), migration (**C**), and invasion (**D**) were examined as described in Materials and Methods. $CoCl_2$ + Puro represent $CoCl_2$ (100 μM) plus puromycin (10 μM). The bars represent the means ± SD of three independent experiments. Statistical significance was tested using one-way ANOVA followed by a Students' *t*-test. $^*P<0.05$ versus control buffer.

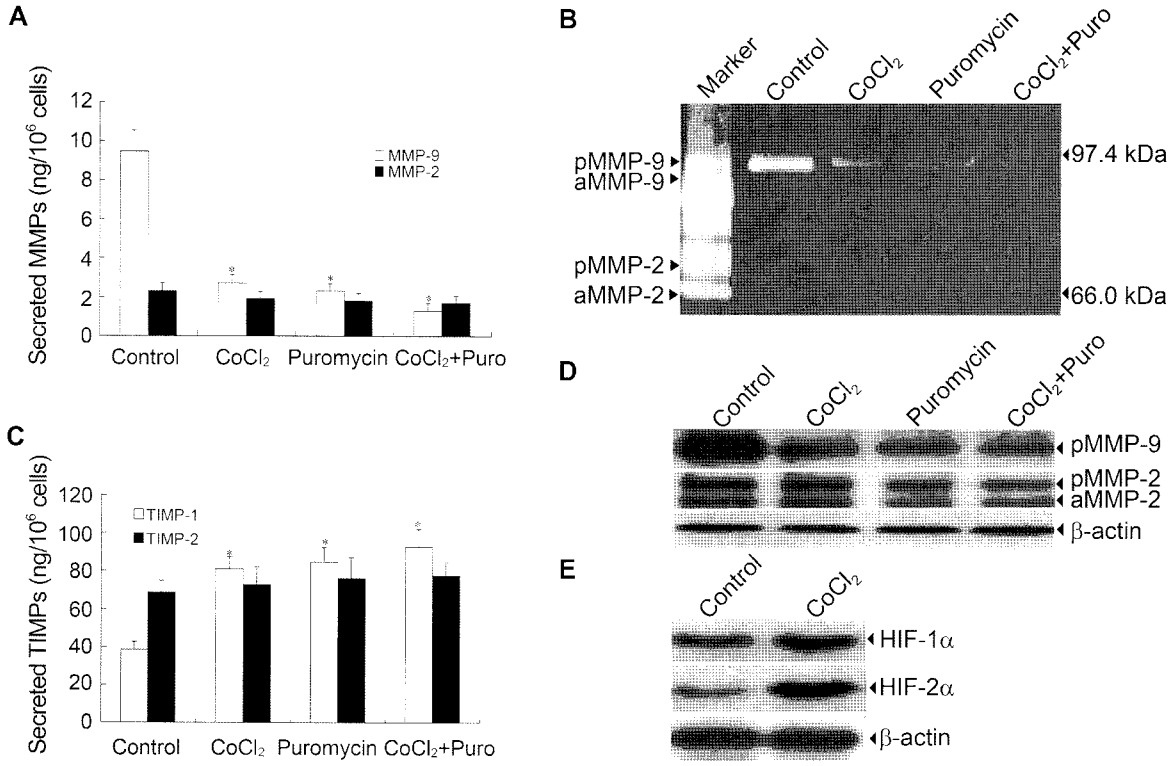

Fig. 3. The effects of $CoCl_2$ and puromycin on the secretion and expression of MMPs and TIMPs.
A, C. Cells were incubated in serum-free and phenol-red-free RPMI-1640 for 12 h. Next, the cells were incubated for 12 h following the addition of a control buffer (Control), $CoCl_2$ (100 μM), puromycin (10 μM), or $CoCl_2$ (100 μM) plus puromycin (10 μM) ($CoCl_2$ + Puro). The media were quantitatively assayed by enzyme immunoassay. Bars represent the means ± SD of three independent experiments. *P<0.05 versus control buffer. **B.** Gelatin zymography of MMP-2 and MMP-9 in the culture medium of PC-3 cells. Equal amounts of proteins (10 mg/lane) from the supernatants were loaded into each lane. **D.** The levels of MMP-2 and MMP-9 expression were determined by Western blotting with MMP-2 and MMP-9 specific antibodies. **E.** The levels of HIF-1α and HIF-2α expression were determined by Western blotting with HIF-1α and HIF-2α specific antibodies. Protein expression levels of β-actin in the cell lysates were used as a control.

Cobalt Chloride Modulates MMP-9 Protein Levels Through PSA Expression

MMPs have been regarded as major critical molecules that assist tumor cells during invasion, and there is a clear connection between MMPs and cell invasion [14]. Because PSA was downregulated by $CoCl_2$, we used an enzyme immunoassay to examine the effect of $CoCl_2$ and puromycin on the secretion of MMP-2 and MMP-9. As shown in Fig. 3A, $CoCl_2$ (100 μM) and puromycin (10 μM) suppressed the secretion of MMP-9 by 72% and 76%, respectively, when compared with the addition of a control buffer, whereas MMP-2 was not affected. Furthermore, treatment with a combination of $CoCl_2$ (100 μM) and puromycin (10 μM) resulted in an enhanced suppressive effect (87%) on the secretion of MMP-9 (Fig. 3A). The profiles of the MMPs in the media were also semiquantitatively assayed by gelatin zymography. Consistent with the results of the enzyme immunoassay, $CoCl_2$ and puromycin suppressed the secretion of MMP-9 (Fig. 3B). Moreover, combined treatment of PC-3 cells with $CoCl_2$ and puromycin almost completely suppressed the secretion of MMP-9 (Fig. 3B). Finally, treatment with $CoCl_2$ and puromycin suppressed the expression of MMP-9 by PC-3 cells, whereas it had no effect on the expression of MMP-2 (Fig. 3D). In many cell types, the enzyme activities of MMP-2 and MMP-9 are activated by hypoxia regulated with a time-dependent manner of HIF-1α expression. The activities of MMPs are controlled by the four natural tissue inhibitors of metalloproteinases (TIMPs). TIMP-2 interacts with MMP-2 and TIMP-1 forms a complex with MMP-9. This implies that the biological processes involving MMPs are dependent on balances between MMPs and TIMPs. As shown in Fig. 3C, TIMP-1 significantly increased in $CoCl_2$-induced hypoxia in PC-3 cells. However, TIMP-2 was not affected by $CoCl_2$-induced hypoxia. Through this experiment, we reconfirmed the decreased secretion of MMP-9 by $CoCl_2$-induced hypoxia in PC-3 cells. Zhao *et al.* [28] showed that hypoxia inhibits the production of MMP-9 and the migration of human dendritic cells. Taken together, we confirmed that hypoxia specifically downregulates the expression of PSA, and PSA modulates the expression and secretion of MMP-9, independent of the expression of HIF-1 in PC-3 cells (Figs. 1 and 3E). This illustrates the potential for disparate results when using gas hypoxia or chemical inducers of HIF-1 such as $CoCl_2$. These results indicate that $CoCl_2$ has a direct effect on the secretion and

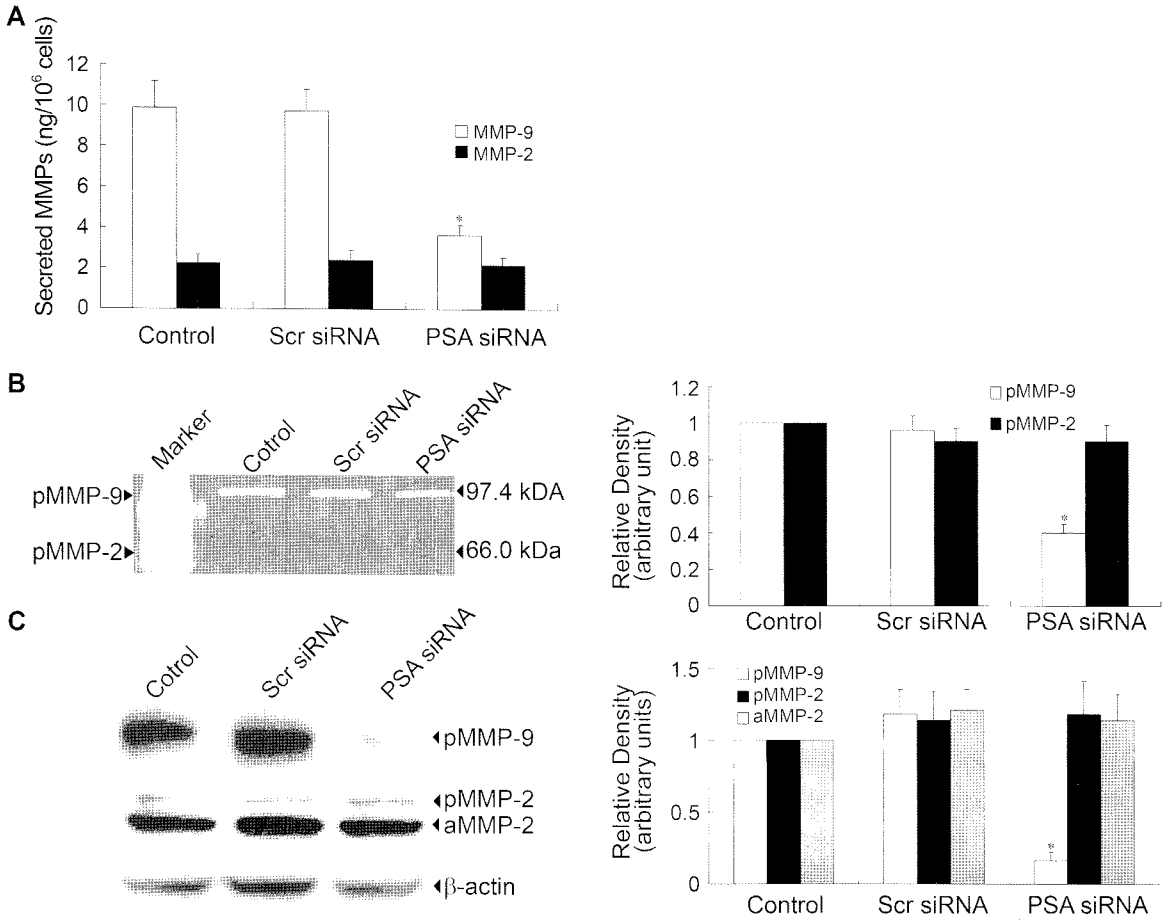

Fig. 4. Effects of PSA siRNA on the secretion and expression of MMP-2 and MMP-9.
A. Cells were incubated in serum-free and phenol-red-free RPMI-1640 for 12 h. Next, the cells were incubated for 12 h following the addition of control buffer (Control), scramble RNAs (Scr siRNA, 60 nM), or PSA siRNA (60 nM). Bars represent the means ± SD of three independent experiments. Statistical significance was tested using one-way ANOVA followed by the Student *t* test. $^*P<0.05$ versus control buffer. **B**. Gelatin zymography of MMP-2 and MMP-9 in the culture medium of PC-3 cells. Equal amounts of proteins (10 mg/lane) from supernatants were loaded into each lane. **C**. MMP-2 and MMP-9 expression was assessed using Western blot 48 h after transfection with Scr siRNA (60 nM) and PSA siRNA (60 nM). The protein expression levels of β-actin in the cell lysates were used as a control.

expression of MMP-9, as well as an indirect effect that is exerted *via* PSA suppression. Furthermore, our results suggest that $CoCl_2$ and puromycin inhibit cell migration and invasion, in part by inhibiting MMP-9 production in PC-3 cells.

PSA May be Involved in Angiogenesis

To elucidate the molecular mechanisms involved in the suppressive effects of PSA siRNA on PC-3 cell migration and invasion, we examined the expression and enzymatic activities of MMP-2 and MMP-9. As shown in Fig. 4A, PSA siRNA resulted in the suppression of approximately 64% of the MMP-9 produced by PC-3 cells, whereas it had no effect on MMP-2. Consistent with the enzyme immunoassay data, gelatin zymography revealed that PSA siRNA reduced the level of MMP-9 that was secreted by the PC-3 cells (Fig. 4B). The levels of MMP-2 and MMP-9 were also determined by Western blot analysis. PSA siRNA suppressed the expression of MMP-9 by PC-3 cells, whereas MMP-2 was not affected (Fig. 4C). These results indicate that PSA siRNA has a negative effect on cancer cell migration and invasion through a mechanism that involves downregulation of MMP-9 expression and secretion of PC-3 cells. These findings suggest that promotion of PSA-mediated suppression of MMP-9 may provide new strategies for the prevention of cancer metastasis. Miyashita *et al.* [21] reported that PILSAP inhibited the proliferation, migration, and network formation of ECs *in vitro* and angiogenesis *in vivo*. Therefore, PSA may play a role in angiogenesis similar to that of PILSAP. Furthermore, the results of our study demonstrated that PSA significantly inhibited the proliferation, migration, and invasion of PC-3 cells. This inhibition of migration and invasion may be associated with the observed decrease in MMP-9 protease activity.

In summary, we have demonstrated that the PSA gene was downregulated in response to treatment with $CoCl_2$

and that PSA is involved in the proliferation, migration, and invasion of PC-3 cells, which are important determinants of metastasis, *via* a mechanism that involves MMP-9 modulation.

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References

1. Akada, T., T. Yamazaki, H. Miyashita, O. Niizeki, M. Abe, A. Sato, S. Satomi, and Y. Sato. 2002. Puromycin insensitive leucyl-specific aminopeptidase (PILSAP) is involved in the activation of endothelial integrins. *J. Cell Physiol.* **193:** 253–262.
2. Bergers, G. and L. E. Benjamin. 2003. Tumorigenesis and the angiogenic switch. *Nat. Rev. Cancer* **3:** 401–410.
3. Bhagwat, S. V., J. Lahdenranta, R. Giordano, W. Arap, R. Pasqualini, and L. H. Shapiro. 2001. CD13/APN is activated by angiogenic signals and is essential for capillary tube formation. *Blood* **97:** 652–659.
4. Brahimi-Horn, C., E. Berra, and J. Pouyssegur. 2001. Hypoxia: The tumor's gateway to progression along the angiogenic pathway. *Trends Cell Biol.* **11:** S32–S36.
5. Bunn, H. F. and R. O. Poyton. 1996. Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76:** 839–885.
6. Caudroy, S., M. Polette, B. Nawrocki-Raby, J. Cao, B. P. Toole, S. Zucker, and P. Birembaut. 2002. EMMPRIN-mediated MMP regulation in tumor and endothelial cells. *Clin. Exp. Metastasis* **19:** 697–702.
7. Constam, D. B., A. R. Tobler, A. Rensing-Ehl, I. Kemler, L. B. Hersh, and A. Fontana. 1995. Puromycin-sensitive aminopeptidase. Sequence analysis, expression, and functional characterization. *J. Biol. Chem.* **270:** 26931–26939.
8. Du, R., K. V. Lu, C. Petritsch, P. Liu, R. Ganss, E. Passegue, *et al.* 2008. HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell* **13:** 206–220.
9. Egeblad, E. and Z. Werb. 2002. New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* **2:** 161–174.
10. Folkman, J. and M. Klagsbrun. 1987. Angiogenic factors. *Science* **235:** 442–447.
11. Folkman, J. and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* **267:** 10931–10934.
12. Genbacev, O., Y. Zhou, J. W. Ludlow, and S. J. Fisher. 1997. Regulation of human placental development by oxygen tension. *Science* **277:** 1669–1672.
13. Griffith, E. C., Z. Su, B. E. Turk, S. Chen, Y. H. Chang, and J. O. Liu. 1997. Methionine aminopeptidase (type 2) is the common target for angiogenesis inhibitors AGM-1470 and ovalicin. *Chem. Biol.* **4:** 461–471.
14. Gupta, G. P. and J. Massague. 2006. Cancer metastasis: Building a framework. *Cell* **127:** 679–695.
15. Hersh, L. B., T. E. Smith, and J. F. McKelvy. 1980. Cleavage of endorphins to des-Tyr endorphins by homogeneous bovine brain aminopeptidase. *Nature* **286:** 160–162.
16. Kakuta, H., A. Tanatani, K. Nagasawa, and Y. Hashimoto. 2003. Specific nonpeptide inhibitors of puromycin-sensitive aminopeptidase with a 2, 4 (1H, 3H)-quinazolinedione skeleton. *Chem. Pharm. Bull.* **51:** 1273–1282.
17. Klagsbrun, M. and P. A. D'Amore. 1991. Regulators of angiogenesis. *Annu. Rev. Physiol.* **53:** 217–239.
18. Komoda, M., H. Kakuta, H. Takahashi, Y. Fujimoto, S. Kadoya, F. Kato, and Y. Hashimoto. 2001. Specific inhibitor of puromycin-sensitive aminopeptidase with a homophthalimide skeleton: Identification of the target molecule and a structure-activity relationship study. *Bioorg. Med. Chem.* **9:** 121–131.
19. Lockhart, D. J. and E. A. Winzeler. 2000. Genomics, gene expression and DNA arrays. *Nature* **405:** 827–836.
20. Marchio, S., J. Lahdenranta, R. O. Schlingemann, D. Valdembri, P. Wesseling, M. A. Arap, *et al.* 2004. Aminopeptidase A is a functional target in angiogenic blood vessels. *Cancer Cell* **5:** 151–162.
21. Miyashita, H., T. Yamazaki, T. Akada, O. Niizeki, M. Ogawa, S. Nishikawa, and Y. Sato. 2002. A mouse orthologue of puromycin-insensitive leucyl-specific aminopeptidase is expressed in endothelial cells and plays an important role in angiogenesis. *Blood* **99:** 3241–3249.
22. Saric, T., C. I. Graef, and A. L. Goldberg. 2004. Pathway for degradation of peptides generated by proteasomes: A key role for thimet oligopeptidase and other metallopeptidases. *J. Biol. Chem.* **279:** 46723–46732.
23. Sato, Y. 2004. Role of aminopeptidase in angiogenesis. *Biol. Pharm. Bull.* **27:** 772–776.
24. Schugart, R. C., A. Friedman, R. Zhao, and C. K. Sen. 2008. Wound angiogenesis as a function of tissue oxygen tension: A mathematical model. *Proc. Natl. Acad. Sci.* U.S.A. **105:** 2628–2633.
25. Stoltze, L., M. Schirle, G. Schwarz, C. Schroter, M. W. Thompson, L. B. Hersh, *et al.* 2000. Two new proteases in the MHC class I processing pathway. *Nat. Immunol.* **1:** 413–418.
26. Su, Y., J. Zhang, and R. Xiu. 2006. Down-regulation of hepatocyte growth factor mRNA in rat cardiac myocytes under hypoxia mimicked by cobalt chloride. *Clin. Hemorheol. Microcirc.* **34:** 201–206.
27. Yamazaki, T., T. Akada, O. Niizeki, T. Suzuki, H. Miyashita, and Y. Sato. 2004. Puromycin-insensitive leucyl-specific aminopeptidase (PILSAP) binds and catalyzes PDK1, allowing VEGF-stimulated activation of S6K for endothelial cell proliferation and angiogenesis. *Blood* **104:** 2345–2352.
28. Zhao, P., X. G. Li, M. Yang, Q. Shao, D. Wang, S. Liu, *et al.* 2008. Hypoxia suppresses the production of MMP-9 by human monocyte-derived dendritic cells and requires activation of adenosine receptor A2b *via* cAMP/PKA signaling pathway. *Mol. Immunol.* **45:** 2187–2195.