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ARTICLES

Transforming Growth Factor-β1-Dependent Activation of Smad2/3 and Up-Regulation of PAI-1 Expression Is Negatively Regulated by Src in SKOV-3 Human Ovarian Cancer Cells

Kiyoshi Wakahara,[1] Hiroshi Kobayashi,[2]* Tatsuo Yagyu,[1] Hidenori Matsuzaki,[1] Toshiharu Kondo,[3] Noriyuki Kurita,[4] Hideo Sekino,[4] Kiyokazu Inagaki,[1] Mika Suzuki,[2] Naohiro Kanayama,[2] and Toshihiko Terao[2]

[1]NetForce Co. Ltd., Taiko 3-1-18, Nakamura, Nagoya, Aichi 453-0801, Japan
[2]Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, Handayama 1-20-1, Hamamatsu, Shizuoka 431-3192, Japan
[3]Computer Technology Integration (CTI) Co. Ltd., Meieki-minami 1-27-2, Nakamura, Nagoya, Aichi 450-0003, Japan
[4]Department of Knowledge-Based Information Engineering, Toyohashi University of Technology, Tempaku-cho, Toyohashi 441-8580, Japan

Abstract The net balance between urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type-1 (PAI-1) has been implicated in tumor cell invasion and metastasis. To elucidate the mechanism of the transforming growth factor-β1 (TGF-β1)-dependent up-regulation of PAI-1 expression, we investigated which signaling pathway transduced by TGF-β1 is responsible for this effect. Here, we show (1) nontoxic concentrations of TGF-β1 up-regulates uPA expression in HRA and SKOV-3 human ovarian cancer cells, (2) TGF-β1 activates Smads (phosphorylation of Smad2 and nuclear translocation of Smad3) and subsequently up-regulates PAI-1 expression in HRA cells, whereas TGF-β1 neither activates Smads nor up-regulates PAI-1 in SKOV-3 cells, (3) pharmacological Src inhibitor PP2 or antisense (AS) c-Src oligodeoxynucleotide (ODN) treatment significantly induces TGF-β1-dependent activation of Smads, leading to PAI-1 synthesis, compared with controls, in SKOV-3 cells, (4) combination of TGF-β1 and PP2, which activates PAI-1 expression and reduces uPA expression in SKOV-3, results in decreased invasiveness, (5) pharmacological inhibitors for mitogen-activated protein kinase (MAPK) (PD98059) and phosphoinositide-3-kinase (PI3K) (LY294002 and wortmannin) or AS-PI3K ODN transfection do not affect TGF-β1-induced Smad signaling and up-regulation of PAI-1 expression in SKOV-3 cells pretreated with PP2, and (6) the induction of PAI-1 protein was partially inhibited by an inhibitor of Sp1-DNA binding, mithramycin, implicating, at least in part, Sp1 in the regulation of this gene by TGF-β1. In conclusion, TGF-β1-dependent activation of Smad2/3, leading to PAI-1 synthesis, may be negatively regulated by Src, but not its downstream targets MAPK and PI3K in SKOV-3 cells. These data also reflect the complex biological effect of uPA-PAI-1 system. J. Cell. Biochem. 93: 437–453, 2004.

Key words: invasion; ovarian cancer; PAI-1; Src; signal transduction; uPA

Abbreviations used: AS, antisense; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; ODN, oligodeoxynucleotide; PAI-1, plasminogen activator inhibitor type-1; PI3K, phosphoinositide-3-kinase; TGF-β, transforming growth factor-β; uPA, urokinase-type plasminogen activator.

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*Correspondence to: Hiroshi Kobayashi, Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, Handayama 1-20-1, Hamamatsu, Shizuoka, 431-3192, Japan.

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The processes of ovarian cancer dissemination are characterized by altered local proteolysis, cellular proliferation, cell attachment, and invasion, suggesting that the balance between urokinase-type plasminogen activator (uPA) and its specific inhibitor plasminogen activator inhibitor type-1 (PAI-1) could be involved in the pathogenesis of peritoneal dissemination [Hirashima et al., 2003]. uPA is a serine protease associated with various pathological conditions including tumor invasion and metastasis [Muehlenweg et al., 2001].

One of the factors regulating the metastatic process is considered to be transforming growth factor-β (TGF-β), which is a multifunctional cytokine that elicits numerous cellular effects pertinent to the metastatic process [Hirashima et al., 2003]. TGF-β regulates a wide range of physiological and pathological cellular processes, including cell growth, differentiation, invasion, migration, mesenchymal transition, extracellular matrix (ECM) synthesis, and cell death in many cell types including ovarian cancer cells [Kim and Joo, 2002]. TGF-β family members signal through transmembrane Ser-Thr kinase receptors that directly regulate the intracellular Smad pathway, leading to the induction of PAI-1 expression [Macias-Silva et al., 1996; Nakao et al., 1997; Massagué and Wotton, 2000; Miyazono et al., 2001; ten Dijke et al., 2002]. These activated receptor-regulated Smad2/3 can form a hetero-oligomeric complex with the co-Smad, Smad4, which is then translocated into the nucleus to induce transcriptional activation [Lagna et al., 1996; Derynck and Feng, 1997; Heldin et al., 1997; Nakao et al., 1997]. In addition, Smad3 and Smad4 bind directly to a TGF-β-responsive sequence in the human PAI-1 promoter that is associated with the ability to mediate strong transcriptional activation of this gene by TGF-β [Song et al., 1998]. TGF-β-responsive element in the human PAI-1 promoter contains two Sp1 binding sites.

Our laboratory has extensively studies the cellular mechanism(s) of the TGF-β-induced uPA-dependent tumor invasion and metastasis [Hirashima et al., 2003]. In a well-characterized human ovarian cancer HRA cell model using antisense (AS) strategies and pharmacological inhibitors, we examined the distinct TGF-β signaling events [Hirashima et al., 2003; Kobayashi et al., 2003]. We have previously explored the signal transduction mechanisms downstream of the TGF-β receptors that result in uPA up-regulation and cell invasion. However, mechanisms by which TGF-β up-regulates uPA expression are heterogeneous and depend upon the particular complement of signaling molecules expressed within a given cell type. The previous data [Tanaka et al., 2003] support a role for TGF-β1 activation of two distinct pathways (Src-mitogen-activated protein kinase (MAPK)-phosphoinositide-3-kinase (PI3K)-NF-κB-dependent and Src-MAPK-AP-1-dependent) for TGF-β1-dependent uPA up-regulation and promotion of invasion. However, we have no information on TGF-β1-dependent Smad activation and subsequent up-regulation of PAI-1 expression. The role that TGF-β/Smad signaling plays in ovarian cancer cell invasion is also unclear.

In the present studies, we have used the TGF-β-sensitive HRA cells and TGF-β-insensitive SKOV-3 cells on regulation of PAI-1 expression to determine the TGF-β/Smad signaling pathway, in relation with the status of Src, MAPK, PI3K, and Smads. The key experiments were repeated with HRA and SKOV-3 ovarian cancer cell lines. These findings have important implications for our understanding of the role of TGF-β in regulating uPA and PAI-1 expression and subsequent invasion. We report here, for the first time, the potent ability of c-Src kinase to affect TGF-β/Smad signaling, to regulate the effects of TGF-β-dependent up-regulation of PAI-1 expression, and to modulate invasiveness in ovarian cancer cells.

MATERIALS AND METHODS

Materials

LipofectAMINE Plus reagent was purchased from Life Technologies, Inc. (Rockville, MD). Boyden-type cell invasion chambers (BioCoat Matrigel^{TM} invasion chambers) were obtained from Collaborative Biomedical (Franklin Lakes, NJ). Ultrapure natural human TGF-β1 was from Genzyme (Cambridge, MA) and R&D Systems (Minneapolis, MN). The antibodies against uPA (#3689 [recognizes uPA B-chain] and #3471 [reacts with uPA A-chain; interferes with binding of uPA to its receptor]) and PAI-1 (#3785) were obtained from American Diagnostics (Greenwich, CT). Peroxidase-conjugated secondary antibodies were from Dako (Copenhagen, Denmark) and Santa Cruz Biotechnology (Santa Cruz, CA); antibody to phospho-Smad2

was from Upstate Biotechnology (Lake Placid, NY); and antibodies to Smad2 and Smad3 were from Zymed Laboratories (South San Francisco, CA). The nude mice (Balb-c, nu/nu) were obtained from SLC (Hamamatsu, Japan). Anti-actin antibody was from CosmoBio (Tokyo, Japan). Culture media, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Life Technologies, Inc. Tissue culture plastics were purchased from Costar/Corning (Cambridge, MA) and Falcon (Becton Dickinson and Co., Bedford, MA). Bovine serum albumin (BSA), Tris-base, dithiothreitol (DTT), phenylmethylsulfonyl fluoride, and ammonium persulfate were commercially obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, bisacrylamide, and PVDF membrane were from Bio-Rad. X-ray film was purchased from Kodak Co. The enhanced chemiluminescence (ECL) was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). All other chemicals were analytical grade.

Pharmacological Inhibitors

The inhibitors were dissolved in Me_2SO (cell culture grade; Sigma) and used in the following concentrations: wortmannin (100 nM, specific inhibitor of PI3K), LY294002 (10 μM, specific inhibitor of the p110 catalytic subunit of PI3K), SB202190 (35 μM, P38 kinase inhibitor), and PD98059 (50 μM, specific inhibitor of MEK). All of the inhibitors except wortmannin (Sigma) were obtained form Calbiochem (La Jolla, CA). The inhibitors diluted in normal growth medium were added to wells containing confluent cells and incubated for 30 min to 1 h. TGF-β1 (10 ng/ml) was added to serum-free medium containing the respective inhibitors and incubated for indicated periods of time, after which time the conditioned medium and cells were separately collected and the cells were counted. The samples were stored at −80°C until measured. Me_2SO (0.05% v/v) diluted in medium was used as a negative control.

Cell Culture

The ovarian cancer cell line, HRA, was grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (v/v) FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere with constant humidity [Suzuki et al., 2003]. SKOV-3 ovarian cancer cells were obtained from American Type Culture Collection. The cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS and antibiotics. TGF-β1 (10 ng/ml) was added either alone or in combination in cancer cells preincubated for 30 min to 1 h with pharmacological inhibitors.

Preparation of Nuclear and Whole Cell Extracts (Total Cell Lysates)

For whole cell extraction, cells were lysed in 250 mM Tris-HCl, pH 7.5, by three cycles of freezing and thawing, followed by clearing the lysate by centrifugation for 5 min at 13,000 rpm at 4°C. Nuclear extracts were prepared essentially as described [Blumenthal et al., 1999]. Total protein amount in the extracts was measured using the Bio-Rad Bradford reagent.

Northern Blot Hybridization With cDNA Probes

Total RNA isolations were done using the Trizol reagent (Life Technologies, Inc.). Northern blot hybridization was carried out as described previously [Kobayashi et al., 2002; Suzuki et al., 2002]. Samples of total RNA (10 μg) were separated by electrophoresis through denaturing 1.2% agarose gels containing 1% formaldehyde and transferred onto nylon or nitrocellulose membranes using standard molecular biological techniques. Hybridization was carried out with [α-^{32}P]dCTP by random oligonucleotide priming to specific activities of $0.4–0.9 \times 10^9$ cpm/μg. PAI-1 cDNA was prepared as described [Kobayashi et al., 2002; Suzuki et al., 2002]. Filters were reprobed with the cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to correct for the amount of RNA loaded onto the filters [Kobayashi et al., 2002; Suzuki et al., 2002]. After hybridization, the membranes were washed and exposed on Kodak BioMax MS-1 film at −70°C. Filters were quantitated by scanning densitometry using a Bio-Rad model 620Video Densitometer with one-dimensional Analyst software package for Macintosh.

Preparation of Oligodeoxynucleotides (ODNs) and Lipofection of HRA Cells

AS ODNs were selected for sequence target to c-Src (AS c-Src, 5′-GGG CTT GCT CTT GCT GCT CCC CAT-3′; sense c-Src, 5′-ATG GGG AGC AGC AAG AGC AAG CCC-3′) and PI3K p85 (AS PI3K, 5′-GTA CTG GTA CCC CTC AGC ACT CAT-3′; sense PI3K, 5′-ATG AGT GCT GAG GGG TAC CAG TAC-3′) [Morel et al.,

2002]. The corresponding sense ODN was used as control for each AS ODN. Furthermore, each inverted antisense (iAS) oligonucleotide (iAS c-Src ODN or iAS PI3K ODN) provided additional controls for the vehicle and transfection. The ODNs were synthesized, purified, and modified with phosphorothioate. Oligonucleotides mixed with Lipofectin reagent were incubated for 15 min at room temperature. Thereafter, the oligonucleotides–liposome complexes were then added to cells and washed twice with medium [Felgner et al., 1987]. After 8 h of incubation at 37°C, the cells were collected by centrifugation, washed three times in PBS, resuspended in medium plus 10% FBS, and grown for 48 h.

Western Blot Analysis

Each cell was harvested, and cell pellets were lysed as described above. Centrifuged lysates (50 μg) from each cell were analyzed by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane by semi-dry transfer. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 2% BSA. Blots were probed with the following primary antibodies overnight at 4°C: monoclonal anti-uPA, anti-PAI-1, anti-phospho-Smad2, anti-Smad2, or anti-Smad3 antibodies. This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5,000 for 1 h. Equal loading of protein was confirmed by anti-lamin A/C antibodies (Santa Cruz Biotechnology) for nuclear proteins or anti-actin antibodies (Santa Cruz Biotechnology) for total cell lysates. Detection was achieved by enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to film. Densitometric analysis of Western blots was carried out using the Macintosh Image System. The density of the proteins or RNA analyzed was normalized to the appropriate controls (e.g., lamin A/C, actin, or GAPDH).

Procollagen Deposition Analysis by ^{3}H-Proline Incorporation, SDS–Polyacrylamide Gel Electrophoresis, and Autoradiography

The parental and transfected cells were grown to subconfluency in 12-well plates. The medium was changed to serum-free medium supplemented with 50 μg/ml ascorbic acid for the duration of the experiment. Cells were stimulated with 10 ng/ml TGF-β1, and 6 h later, 0.5 μCi/ml ^{3}H-proline (Amersham Biosciences) was added to the medium for 24 h. Medium was harvested from each well, and cells were trypsinized and counted. Cells were lysed and ECM proteins were fixed in 70% ethanol. Collagen deposition was determined by differential collagenase digestion and liquid scintillation counting [Eickelberg et al., 2001].

Cell Growth Assay

To examine the proliferation of each cell line, 5×10^3 cells were seeded, and the number of cells in each cell line was counted in triplicate after 24 h to assess plating efficiency. Each experiment was done in triplicate.

ECM Invasion Assay

Chemoinvasion assays were carried out in a Boyden chamber as described [Kobayashi et al., 2001]. The upper surface of chamber was precoated with a layer of artificial basement membrane, Matrigel. The cell suspension (1×10^5 cells/well) was added to the upper chamber. The lower chamber was filled with fibroblast-conditioned medium, which acted as a chemoattractant. To measure invasion, incubation was at 37°C for 24 h. The invaded cells in the lower side of the filter were stained with hematoxylin. Triplicate filters were used for each cell type and assay condition, and ten random fields were counted per filter under a microscope (×400). The experiments of inhibition of cell invasiveness were performed as follows: because wortmannin is unstable at 37°C in culture medium, it was added every 6 h to the upper chamber of the Matrigel invasion assay.

Statistics

Data are expressed as mean ± SD of at least three independent triplicate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

TGF-β1-Induced Invasive Response in Ovarian Cancer Cell Lines

TGF-β1-stimulated cell invasion through modified basement membrane matrix (Matrigel) requires uPA activity. We showed previously that TGF-β1 activates predominantly the Src-MAPK-PI3K-dependent signaling pathway for

uPA up-regulation and promotion of invasion [Tanaka et al., 2003]: TGF-β1 stimulates up-regulation of uPA expression in HRA and SKOV-3 ovarian cancer cells (14 and Fig. 1B), as well as promotes HRA cell invasion by approximately fourfold and to a lesser degree induces SKOV-3 cell invasion by 1.4-fold, respectively (Fig. 1A). TGF-β1-stimulated invasion was impaired by pharmacological inhibitors (PP2, PD98059, wortmannin, and LY249002) or AS ODNs to c-Src and PI3K [Tanaka et al., 2003]. Thus, Src signaling, an upstream target of MAPK and PI3K pathways, may contribute to an invasive response in ovarian cancer cells. We have also reported that there are essentially no differences in the signal transduction mechanisms downstream of the TGF-β receptors that result in uPA upregulation and cell invasion between HRA cells and SKOv-3 cells. As shown in Figure 1B, the current study showed that TGF-β1 led to a 5-fold increase in PAI-1-1 protein expression in HRA cells, but failed to stimulate PAI-1 expression in SKOV-3 cells (Fig. 1B). We estimated by Western blot the ratio of optical density of uPA or PAI-1 protein over actin protein under control conditions and after administration of TGF-β1.

Src Is a Potent Inhibitor of the PAI-1 Expression in SKOV-3 Cells

We have been investigating signaling pathways involved in TGF-β1 activation in ovarian cancer cell lines [Kobayashi et al., 2003]. Src activation has been linked to the activation of MAPK and/or PI3K in some systems [Rak et al., 1995]. Our previous data support the critical position of Src upstream of MAPK and PI3K pathways in ovarian cancer cells. We have previously reported [Tanaka et al., 2003] that TGF-β1 can induce phosphorylation of Src, extracellular signal-regulated kinase (ERK)1/2 and Akt, as well as subsequent uPA up-regulation, in HRA and SKOv-3 cells. In the present study, we examined whether TGF-β1 can induce activation of Smads (phosphorylation of Smad2 and nuclear translocation of Smad3), as well as up-regulation of PAI-1 expression and production of collagenous proteins, in HRA and SKOv-3 cells.

To check first whether the up-regulation of PAI-1 expression is induced by TGF-β1 in HRA and SKOv-3 cells, we examined the expression of PAI-1 protein by employing immunoblotting of cell lysates with anti-PAI-1 antibody. We estimated the ratio of optical density of PAI-1 protein over actin protein under control conditions and after administration of TGF-β1 in the presence or absence of inhibitors in two cell lines. When HRA cells are treated with TGF-β1 (10 ng/ml) for 16 h, this cytokine strongly induces PAI-1 protein, as shown in Figure 2A,B, lane 15, whereas SKOV-3 cells failed to stimulate up-regulation of PAI-1 expression in response to TGF-β1 alone (lane 7).

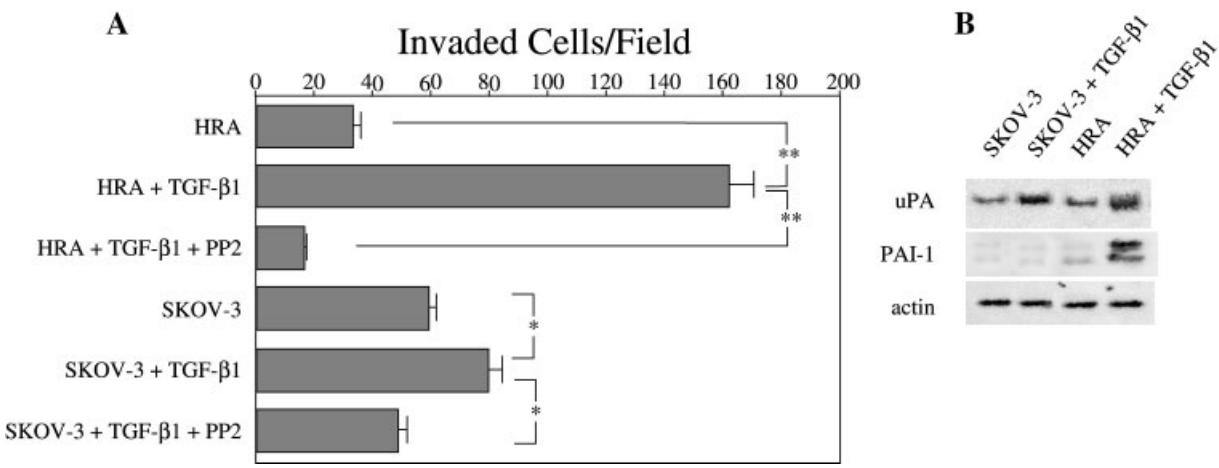

Fig. 1. Effects of transforming growth factor-β1 (TGF-β1) or PP2 on invasive response (**A**) and expression of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type-1 (PAI-1) proteins (**B**). A: A total of 1×10^5 cells were incubated with or without TGF-β1 (10 ng/ml) and/or PP2 (25 μM) in an invasion chamber for 24 h. The data are representative of at least two separate experiments. Values are mean ± SD of two experiments. *, $P < 0.05$; and **, $P < 0.01$. B: Western blot analysis of cell extracts of TGF-β1-treated or untreated cells using antibodies against uPA (**upper panel**), PAI-1 (**middle panel**), or actin (**lower panel**), respectively. Equal loading of protein was confirmed by anti-actin antibodies. The data illustrated are representative of three independent experiments.

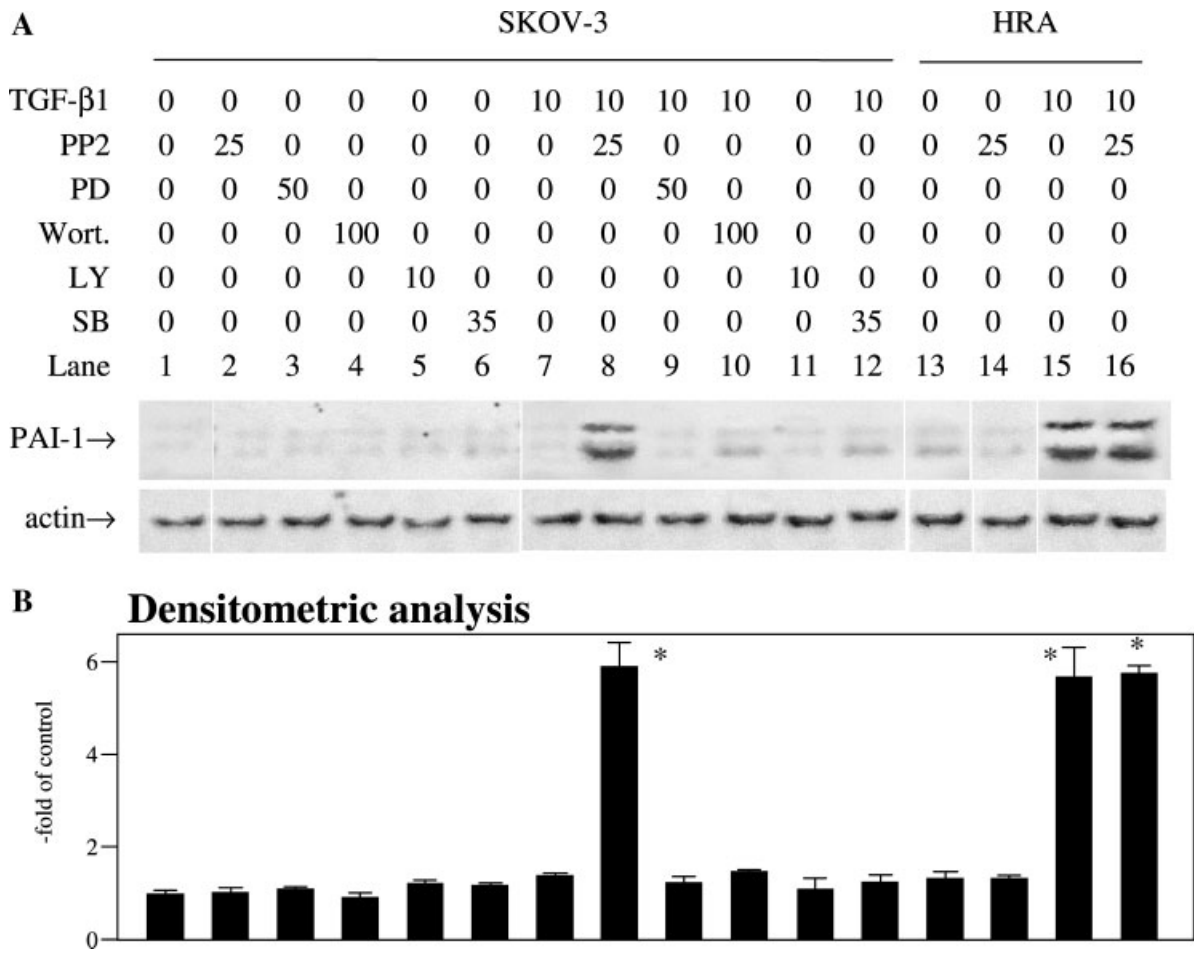

Fig. 2. Effect of pharmacological inhibition of mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K), or p38 MAPK on TGF-β1-dependent expression of PAI-1 protein. **A**: Cells were incubated with compounds with or without TGF-β1 (10 ng/ml) as indicated for 16 h. Total proteins were isolated and electrophoresed. Western blots for PAI-1 (**upper panel**) and actin (**lower panel**) are shown. Equal loading of protein was confirmed by anti-actin antibodies. **B**: Results represent mean ± SD from three independent experiments quantified by densitometry, and the density of PAI-1 was normalized to actin. PAI-1/actin ratio determined by densitometric analysis normalized to 0 ng/ml TGF-β1. *, $P < 0.05$ versus control (**lane 1**).

Since several signals have been demonstrated to regulate TGF-β response, we have measured the ability of pharmacological inhibitors to regulate the expression of PAI-1 protein using this system. We initially tested whether the PAI-1 expression is affected by several pharmacological inhibitors, including Src inhibitor, PP2 (25 μM; lanes 2 and 8), MAPK inhibitor, PD98059 (50 μM; lanes 3 and 9), PI3K inhibitors, wortmannin (100 nM; lanes 4 and 10) and LY249002 (10 μM; lanes 5 and 11), and p38MAPK inhibitor, SB202190 (35 μM; lanes 6 and 12), respectively, in SKOV-3 cells. When SKOV-3 cells were treated simultaneously with PP2, accumulation of PAI-1 protein was significantly enhanced by 10 ng/ml TGF-β1 (Fig. 2, lane 8). Pretreatment of cells with PD98059 (lane 9), wortmannin (lane 10), LY249002 (lane 11), or SB202190 (lane 12) did not affect the TGF-β1-dependent up-regulation of PAI-1 expression. On the other hand, PP2 did not enhance TGF-β1-stimulated up-regulation of PAI-1 protein expression in HRA cells (lane 16 vs. lane 15). If PD98059, wortmannin, LY249002, or SB202190 are individually used as inducer, the PAI-1 production is also resistant to drugs in HRA cells (data not shown). None of these inhibitors alone affected the basal PAI-1 expression (lanes 2–6 and 14). These data suggest that Src signaling may inhibit TGF-β1-stimulated up-regulation of PAI-1 expression in SKOV-3 cells, since MAPK and PI3K are

considered to be downstream targets of Src in these cells [Tanaka et al., 2003].

PP2 Stimulates TGF-β1-Dependent Production of Collagenous Proteins

In the second set of experiments, we tested whether PP2 regulates ECM production in cells in the presence of TGF-β1 by utilizing SKOV-3 and HRA cells. Cell lysates from cells metabolically labeled with ^{3}H-proline were analyzed. HRA cells demonstrate enhanced TGF-β1-induced collagen deposition, irrespective of whether cells were treated with PP2 (Fig. 3, lanes 15 and 16). In contrast, TGF-β1 could not enhance collagenous protein synthesis in SKOV-3 cells. TGF-β1-mediated collagen deposition was stimulated when cells were treated with PP2, but not other pharmacological inhibitors (Fig. 3, lane 8). The collagen production was not significantly induced in cells treated with pharmacologic inhibitors alone without TGF-β1 costimulation (lanes 2–6). Taken together, our results thus far demonstrate that PP2 is a potent stimulator of TGF-β1 signaling (production of PAI-1 and collagenous proteins) in SKOV-3 cells.

Activation of Smad2 Is Enhanced by TGF-β1 in HRA Cells, but not SKOV-3 Cells

Because TGF-β signal transduction is mediated in part by Smad proteins, it is important to evaluate effects of TGF-β on phosphorylation of Smads. In our first experiments on Smad signaling, we treated HRA and SKOV-3 cells

	SKOV-3												HRA			
TGF-β1	0	0	0	0	0	0	10	10	10	10	0	10	0	0	10	10
PP2	0	25	0	0	0	0	0	25	0	00	0	0	0	25	0	25
PD	0	0	50	0	0	0	0	0	50	0	0	0	0	0	0	0
Wort.	0	0	0	100	0	0	0	0	0	100	0	0	0	0	0	0
LY	0	0	0	0	10	0	0	0	0	0	10	0	0	0	0	0
SB	0	0	0	0	0	35	0	0	0	0	0	35	0	0	0	0
Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16

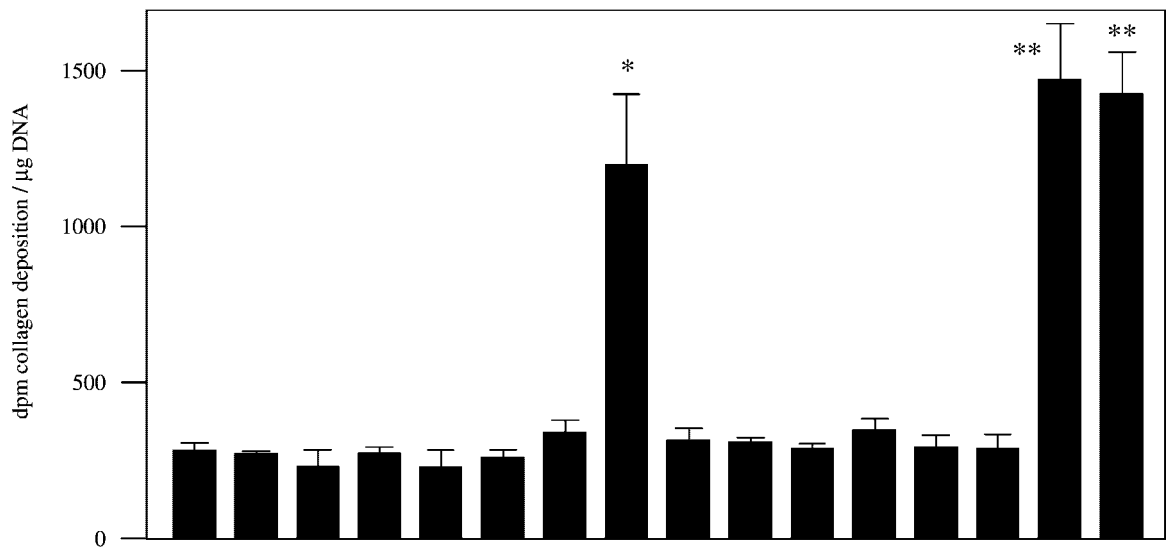

Fig. 3. PP2 inhibits TGF-β1-dependent collagenous protein deposition. Newly synthesized collagenous proteins in SKOV-3 and HRA cells were measured in a [^{3}H]proline incorporation assay after 30 h of 10 ng/ml TGF-β1 stimulation in the presence or absence of pharmacological inhibitors. **A**: Summary of quantitative analysis of the collagenous proteins expressed by cells with and without TGF-β1 (10 ng/ml). Results are presented as dpm collagen deposition/µg DNA. Experiments were performed in triplicate, and data (means ± SD) presented are representative of two independent experiments. *, $P < 0.05$ versus **lane 1** or **lane 13**.

with TGF-β1 for either 20 min or 1 h. Activation of Smad2 was measured with an antibody to phospho-Smad2; this reagent recognizes phosphorylated sites at $Sedr^{465}$ and $Sedr^{467}$, which are direct targets of the TGF-β type I receptor kinase. As shown in Figure 4A,B, within 20 min of treatment, TGF-β1 (10 ng/ml) induced phosphorylation of Smad2 in HRA cells. The amount of total Smad2 did not change in the cells. In contrast to HRA cells, TGF-β1 had no effect in SKOV-3 cells.

Nuclear Translocation of Smad3 by TGF-β1 in Cells Pretreated With or Without Pharmacological Inhibitors

We have next studied the effects of TGF-β1 on nuclear translocation of Smad3, because no antibodies to phospho-Smad3 are available at this time. Smad3 could not be detected in nuclear extracts when SKOV-3 cells had been treated with TGF-β1 alone (Fig. 5A upper panel and B, lanes 3 and 4 vs. lanes 1 and 2). Equal loading of protein was confirmed by anti-actin antibodies for whole cell lysates (middle panel) or anti-lamin A/C antibodies for nuclear proteins (lower panel). Smad3 could be detected in nuclear extracts only when SKOV-3 cells had been co-treated with TGF-β1 and Src inhibitor PP2 (lanes 5 and 6). In addition, TGF-β1 did not stimulate nuclear translocation of Smad3 when SKOV-3 cells were preincubated with PD98059 (lanes 7 and 8), wortmannin (lanes 9 and 10), LY249002 (lanes 11 and 12), or SB202190 (lanes 13 and 14), respectively. Thus, we observed that nontoxic concentrations of TGF-β1 did not induce a rise in the level of nuclear translocation of Smad3 protein nor up-regulation of PAI-1 expression and collagenous protein production in the SKOV-3 cells. In contrast to SKOV-3 cells, TGF-β1 did stimulate nuclear translocation of Smad3 in HRA cells (lanes 17 and 18). PP2 did not augment TGF-β1-induced nuclear translocation of Smad3 (lanes 19 and 20). None of these inhibitors alone affected the Smad 3 translocation in two cell lines (data not shown).

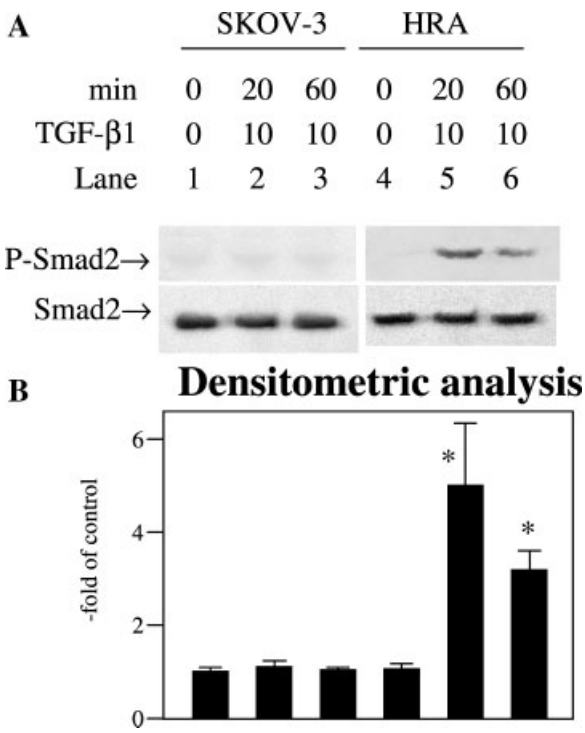

Fig. 4. Activation of Smad2 by TGF-β1. **A**: HRA and SKOV-3 cells were treated with 10 ng/ml TGF-β1 for 20 min or 1 h. Total proteins were obtained and subjected to electrophoresis. Gels were transferred to a membrane and probed with antibody to phospho-Smad2 or total Smad2. **B**: The data illustrated are representative of at least three independent experiments, and mean density ± SD of these bands in cell extract samples in each lane is presented in the graph. *, $P < 0.05$ compared with **lane 4**.

AS c-Src ODN Treatment, but not AS PI3K ODN Treatment, Stimulates TGF-β1-Induced Nuclear Translocation of Smad3, Leading to Up-Regulation of PAI-1 Expression

The role of Src and PI3K was more specifically demonstrated through inhibition with Src and PI3K AS ODN. We evaluated by Western blot analysis, on total cell lysate, the Src and PI3K expression after transfection experiments. As expected, our results (Fig. 6E) provide data on the extent to which Src and PI3K protein expression are reduced by the AS strategy. To further examine the involvement of the Src pathway in TGF-β1-dependent nuclear translocation of Smad3 in SKOV-3 cells, AS ODN targeting of the gene for c-Src, PI3K, or corresponding control ODNs were used to treat cells with subsequent TGF-β1 (10 ng/ml) stimulation. As shown in Figure 6A,B, AS c-Src ODN (lane 6) caused a rise in the level of nuclear translocation of Smad3, apparently through specific reduction in c-Src [Tanaka et al., 2003]. On the other hand, AS PI3K ODN (lanes 7 and 8) and control ODN (lanes 9 and 10) did not affect nuclear translocation. Equal loading of protein was confirmed by anti-actin antibodies for whole cell lysates or anti-lamin A/C antibodies for nuclear proteins (data not shown). Furthermore, none of these ODN

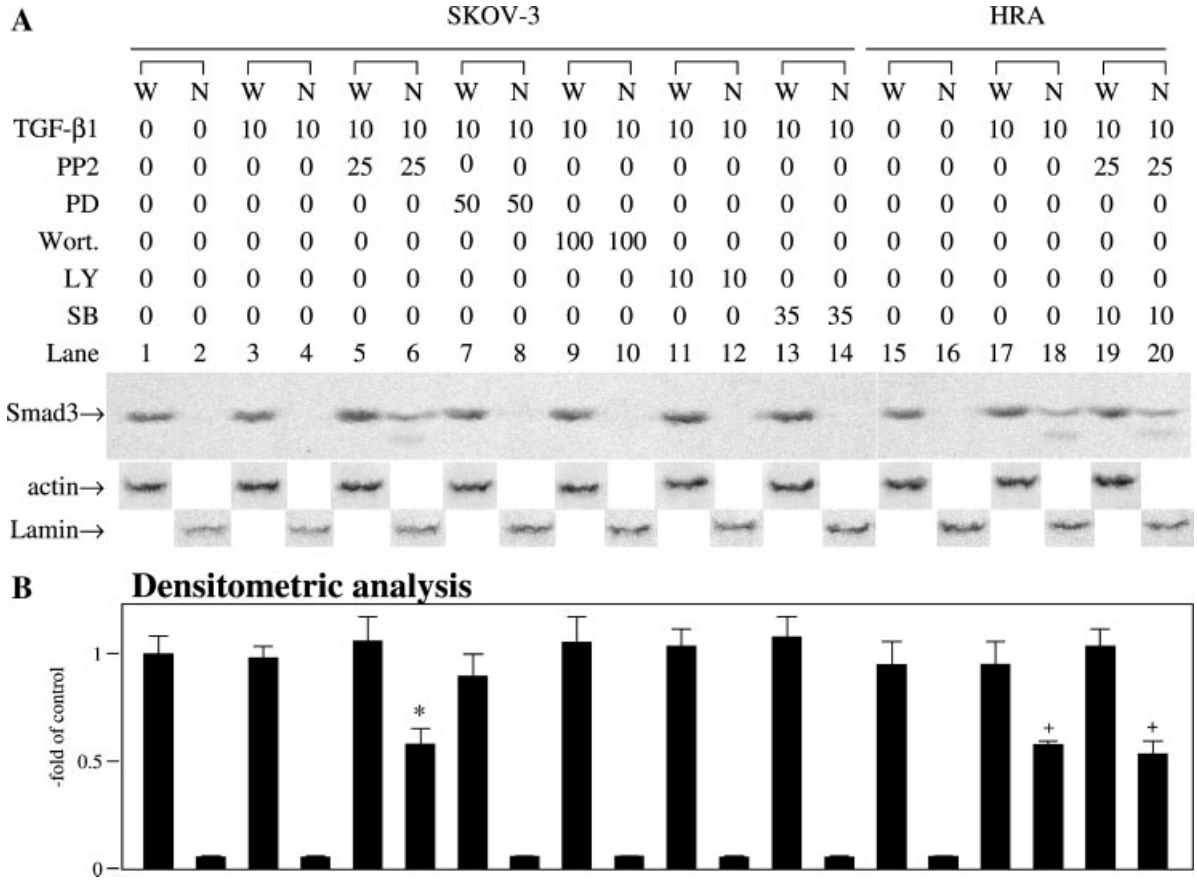

Fig. 5. Nuclear translocation of Smad3 by TGF-β1 in cells pretreated with or without pharmacological inhibitors. **A**: Western blot analysis of whole cell extracts (W) and nuclear extracts (N) of TGF-β1-treated or untreated cells in the presence or absence of each pharmacological inhibitor using antibodies against Smad3 (**upper panel**), actin (**middle panel**), or lamin A/C (**lower panel**). Equal loading of protein was confirmed by anti-actin antibodies for whole cell lysates (W) or anti-lamin A/C antibodies for nuclear proteins (N). **B**: The data illustrated are representative of at least three independent experiments, and mean density ± SD of these bands in cell extract samples in each lane is presented in the graph. The Western blot demonstrating a level of Smad3 in each whole cell extract is a control. *, $P < 0.05$ compared with lane 2 or lane 4 in SKOV-3 cells; and †, $P < 0.05$ compared with lane 16 in HRA cells.

probes alone affected the Smad 3 translocation (data not shown).

Furthermore, to examine the involvement of the c-Src and PI3K pathway in TGF-β1-dependent up-regulation of PAI-1 expression, AS ODN targeting of the gene for c-Src and PI3K was used for further experiments. As shown in Figure 6C,D, AS c-Src ODN (lane 3) did cause a rise in the level of PAI-1 expression, although specific reduction in c-Src protein occurred. In addition, TGF-β1 did not change production of PAI-1 protein in AS PI3K-treated cells (lane 4) or control ODN-treated cells (lane 5). None of these ODN probes alone affected the PAI-1 expression (data not shown). Equal loading of protein was confirmed by anti-actin antibodies.

Pharmacological Inhibition of MAPK or PI3K as Well as AS PI3K ODN Transfection do not Affect TGF-β1-Dependent Nuclear Translocation of Smad3 and Up-Regulation of PAI-1 Expression in SKOV-3 Cells Pretreated With PP2

We examined whether PD98059 can affect TGF-β1-dependent nuclear translocation of Smad3 in SKOV-3 cells preincubated with PP2 (Fig. 7A,B). Incubation of cells with PD98059 (lanes 7 and 8) did not affect the TGF-β1-dependent nuclear translocation of Smad3 protein in PP2-pretreated cells. Similar to PD98059, TGF-β1 also enhanced induction of nuclear translocation in the PP2-treated cells coincubated with PI3K inhibitors [wortmannin (lanes 9 and 10) or LY294002 (lanes 11 and 12)]

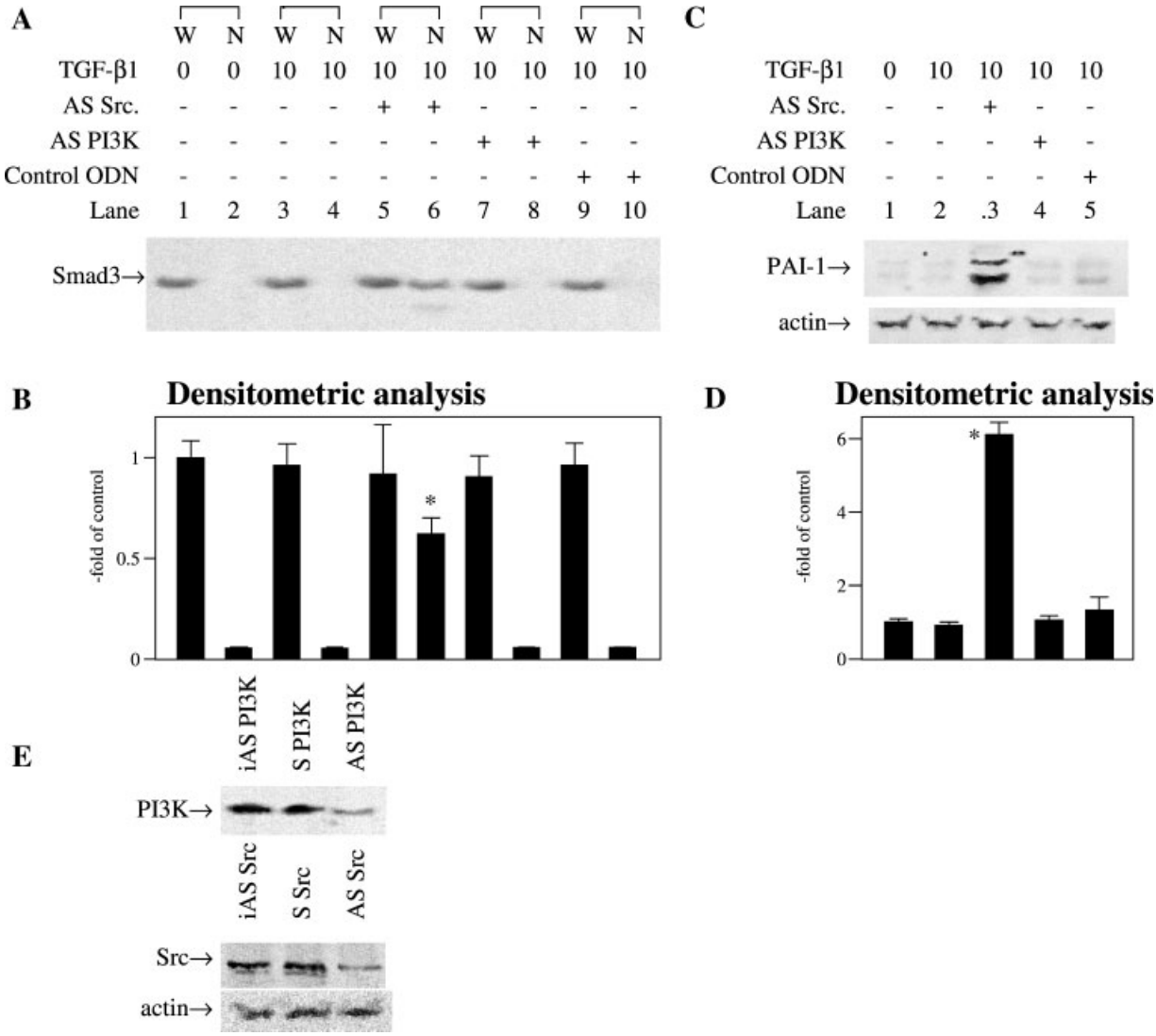

Fig. 6. Nuclear translocation of Smad3 (**A**,**B**) and up-regulation of PAI-1 expression (**C**,**D**) by TGF-β1 in cells treated with AS c-Src ODN or AS PI3K ODN. A: Western blot analysis using antibodies against Smad3, actin (not shown), or Lamin A/C (not shown) of whole cell extracts (W) and nuclear extracts (N) of TGF-β1-treated or untreated cells transiently transfected with AS c-Src ODN or AS PI3K ODN. C: Western blot analysis using antibodies against PAI-1 (**upper panel**) or actin (**lower panel**) of whole cell extracts of TGF-β1-treated or untreated cells transiently transfected with AS c-Src ODN or AS PI3K ODN. B, D: The data illustrated are representative of at least three independent experiments, and mean density (Smad3/actin, Smad3/lamin, or PAI-1/actin) ± SD of these bands in cell extract samples in each lane is presented in the graph. E: Total cell lysates of AS PI3K ODN, S ODN, or iAS PI3K ODN were analyzed by immunoblotting with either anti-PI3K (**upper panel**), anti-Src antibody (**middle panel**), or anti-actin antibody (**lower panel**). *, $P < 0.05$ compared with **lane 2** or **lane 4** in SKOV-3 cells (B); and *, $P < 0.05$ compared with **lane 1** or **lane 2** in SKOV-3 cells (*D*).

or SB202190 (lanes 13 and 14), respectively. PP2 also cause a rise in the level of nuclear translocation in the AS PI3K ODN-treated cells stimulated with TGF-β1 (Fig. 7C,D, lanes 7 and 8). Thus, TGF-β1-stimulated nuclear translocation of Smad3 in the PP2-pretreated cells was not impaired by AS PI3K ODN or control ODNs (sense PI3K ODN). Equal loading of protein was confirmed by anti-actin antibodies for whole cell lysates or anti-lamin A/C antibodies for nuclear proteins (data not shown). Furthermore, TGF-β1 up-regulated PAI-1 protein expression in the PP2-pretreated cells coincubated with PD98059, wortmannin, LY294002, or SB202190 (Fig. 7E,F), as well as in AS PI3K-treated cells (Fig. 7G,H). Equal loading of protein was confirmed by anti-actin antibodies (data not shown).

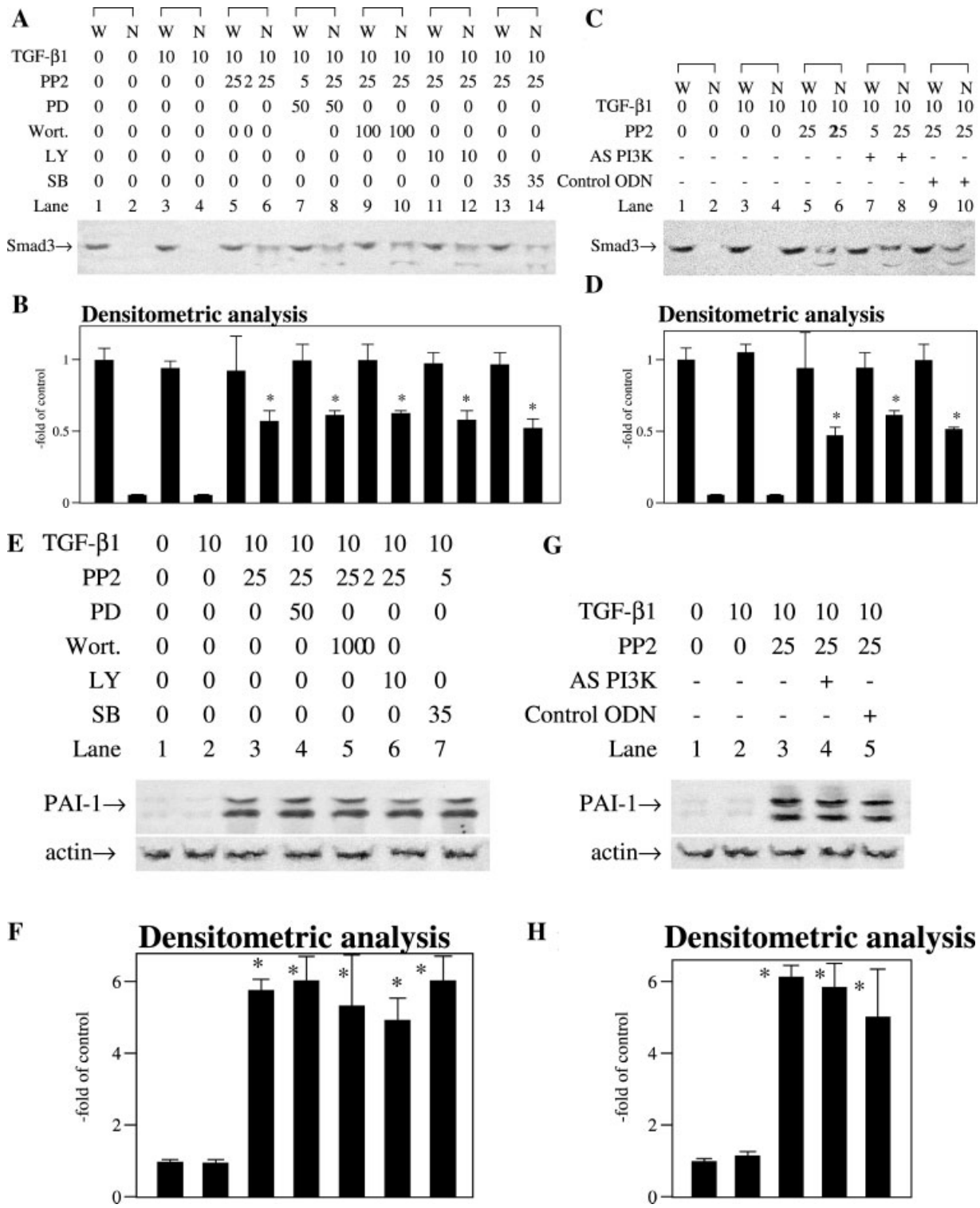

Fig. 7. Nuclear translocation of Smad3 and up-regulation of PAI-1 expression by TGF-β1 in cells pretreated with or without pharmacological inhibitors or antisense (AS) ODNs to Src or PI3K. **A**–**D**: Western blot analysis using antibodies against Smad3, actin (not shown), or lamin A/C (not shown) of whole cell extracts (W) and nuclear extracts (N) of TGF-β1-treated or untreated cells in the presence or absence of each pharmacological inhibitor (A, B) or transiently transfected with AS PI3K ODN or control ODN (C, D). **E**–**H**: Western blot analysis using antibodies against PAI-1 (**upper panel**) or actin (**lower panel**) of cell extracts of TGF-β1-treated or untreated cells in the presence or absence of each pharmacological inhibitor (E and F) or transiently transfected with AS PI3K ODN or control ODN (G and H). B, D, F, and H: The data illustrated are representative of at least three independent experiments, and mean density ± SD of these bands in cell extract samples in each lane is presented in the graph. *, $P < 0.05$ compared with **lanes 2** and **4** (A–D);*, $P < 0.05$ compared with **lanes 1** and **2** (E–H).

Effect of Pharmacological Inhibitors on TGF-β1-Induced Expression of PAI-1 mRNA in Ovarian Cancer Cells Pretreated With PP2

To verify whether the above-mentioned changes of the PAI-1 protein expression were associated with changes of PAI-1 mRNA expression, we estimated by Northern blot the ratio of optical density of PAI-1 mRNA over GAPDH mRNA under control conditions and after administration of TGF-β1 in the presence or absence of PP2, PD98059, wortmannin, LY294002, or SB202190. As shown in Figure 8A,B, 10 ng/ml TGF-β1 produced an increase (peak at 12 h) of PAI-1 mRNA expression in SKOV-3 cells pretreated with PP2 (lane 3), but not PD98059 (lane 8), wortmannin (lane 9), LY294002 (lane 10), or SB202190 (lane 11). Concomitant addition of PP2 plus PD98059 (lane 4), PP2 plus wortmannin (lane 5), PP2 plus LY294002 (lane 6), or PP2 plus SB202190 (lane 7) does not affect the TGF-β1-induced increase of PAI-1 mRNA expression in cells pretreated with PP2, suggesting negative involvement of Src pathway in the TGF-β1-induced PAI-1 synthesis. Downstream targets of Src (MAPK and PI3K pathways) do not affect TGF-β1-dependent PAI-1 expression in these cells. On the other hand, induction of expression of mRNA for PAI-1 was increased by TGF-β1 alone (lane 13) in HRA cells and its increment was not enhanced by co-treatment with PP2 (lane 14). None of these inhibitors alone affected the PAI-1 mRNA expression in two cell lines (data not shown).

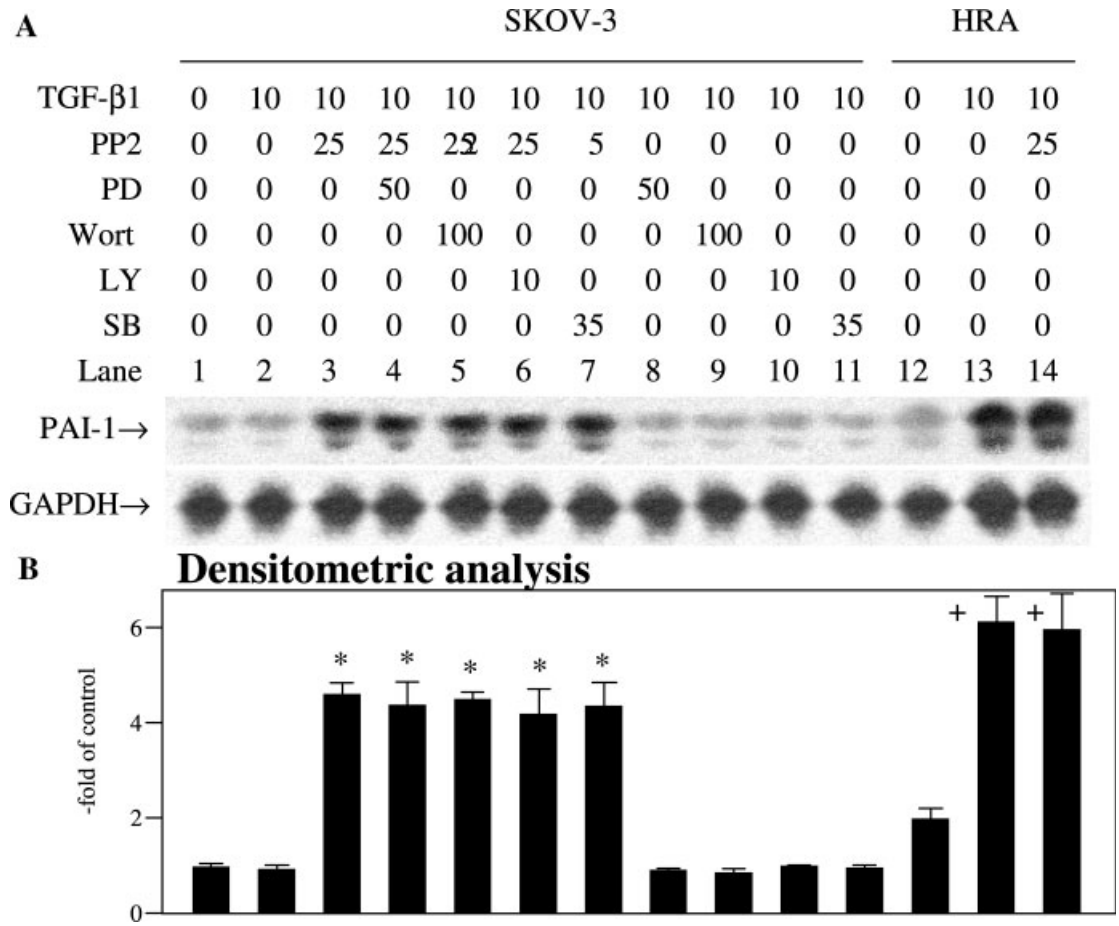

Fig. 8. Effect of PP2 on up-regulation of TGF-β1-induced PAI-1 mRNA expression in ovarian cancer cells. **A**: Cells were incubated for 12 h with TGF-β1 (10 ng/ml) alone or in combination with pharmacological inhibitors as described. Total RNAs were isolated and electrophoresed. Northern blots for PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown. **B**: The data illustrated are representative of at least three independent experiments, and mean PAI-1 mRNA/GAPDH mRNA ratio ± SD of these bands in each lane is presented in the graph. *, $P < 0.05$ compared with **lane 1**; and †, $P < 0.05$ compared with **lane 12**.

Effect of Sp1 Inhibitor on TGF-β1-Induced Expression of the Endogenous PAI-1 Protein

We examined whether the association between endogenous Sp1 and Smad3 is induced by TGF-β1 in SKOV-3 cells. It has been established that an inhibitor of Sp1-DNA binding, mithramycin, blocks TGF-β1 stimulation of PAI-1 protein and that Sp1 interacts with Smad3 and Smad4 [Datta et al., 2000]. We performed immunoblotting experiments with lysates from cells after treating with TGF-β1, PP2, or mithramycin as indicated. TGF-β1 induced the expression of PAI-1 protein in cells pretreated with PP2. Here we show that the induction of PAI-1 protein was partially inhibited by mithramycin (Fig. 9A,B), implicating, at least in part, Sp1 in the regulation of this gene by TGF-β. These data provide insights into the mechanism by which TGF-β up-regulates PAI-1 gene expression by activating Sp1-dependent transcription through the induction of Smad3/Sp1 complex formation. Equal loading of protein was confirmed by anti-actin antibodies (data not shown).

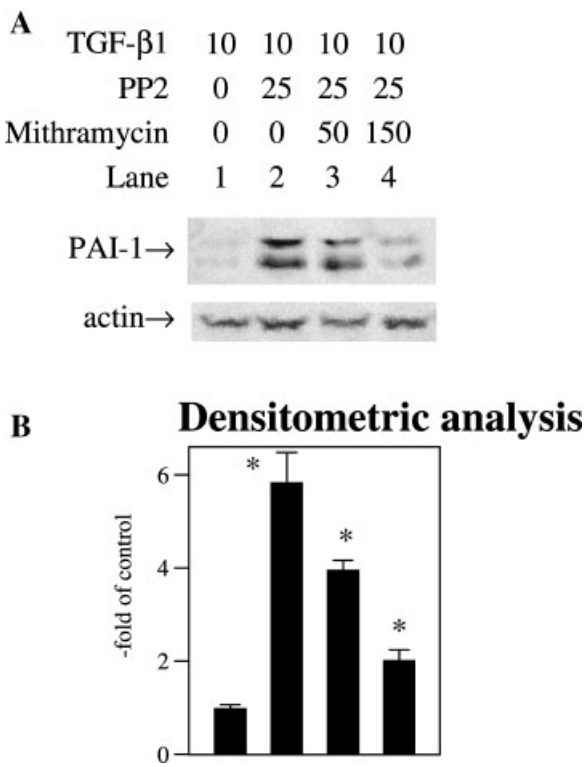

Fig. 9. Effect of Sp1 inhibitor on TGF-β1-induced expression of the endogenous PAI-1 protein. **A**: SKOV-3 cells preincubated with PP2 were treated with or without mithramycin (50 or 150 nM) and TGF-β1 (10 ng/ml). Total cell lysates were subjected to anti-PAI-1 (**upper panel**) or anti-actin (**lower panel**) antibody immunoblotting. **B**: The data illustrated are representative of at least three independent experiments, and mean density ± SD of these bands in cell extract samples in each lane is presented in the graph. *, $P < 0.05$ compared with **lane 1**.

TGF-β1-Induced Invasive Response Is Modulated by PP2

We finally examined the biological phenotype of invasion and the activation or inhibition of the Src signaling pathways studied (Fig. 1A). The combination of TGF-β1 and PP2 significantly reduced uPA expression in HRA cells (data not shown), which results in decreased invasiveness. In addition, exposure of SKOV-3 cells with TGF-β plus PP2 reduced uPA expression and markedly enhanced PAI-1 expression, as well as reduced invasion.

DISCUSSION

There are some conflicting data on biological functions of TGF-β. In general, TGF-β-signaling pathway has been implicated in growth inhibition and tumor suppression [Moustakas et al., 2002]. It has been reported that TGF-β inhibits MAPK activity in certain cells and specifically induces degradation of activated Src kinase [Yue and Mulder, 2001]. In contrast, the previous study provides evidence that TGF-β in HRA and SKOv-3 ovarian cancer cells [Tanaka et al., 2003] as well as HaCaT and MDCK cells induces a rapid and transient increase in Src kinase activity. On the other hand, TGF-β has been reported to negatively regulate Src kinases in HepG2 and PC3 carcinoma cells [Yue and Mulder, 2001]. Thus, TGF-β may play a dual role in cancer biology. TGF-β enhances the invasive properties of ovarian cancer cells, most likely through up-regulation of cellular matrix metalloproteinases [Rodriguez et al., 2001] and uPA [Kobayashi et al., 2003; Tanaka et al., 2003]. However, some cancer cells, including melanoma cells, are invasion inhibited by TGF-β1 [Woodward et al., 2002]. Although the reason for the differing results remains unknown, these differences may depend on a cell type, the stage of differentiation, and culture conditions.

The ability of TGF-β to promote ovarian cancer cell invasion is likely to be mediated by the net balance between uPA and PAI-1 in response to TGF-β treatment. Our previous observation showed that the TGF-β1-dependent invasiveness of HRA cells involved uPA-dependent and

matrix metalloproteinase (MMP)-independent pathways. However, little is known about TGF-β-dependent signaling pathways on PAI-1 expression in ovarian cancer cells. The data presented here extend our previous studies [Kobayashi et al., 2003; Tanaka et al., 2003]. TGF-β1 is able to stimulate up-regulation of uPA in two cell lines [Tanaka et al., 2003]. Up-regulation of PAI-1 expression in response to TGF-β1 is observed only in HRA. The HRA cells can increase mRNA levels of endogenous PAI-1 in response to TGF-β1, demonstrating that these cells maintain a functional TGF-β/Smad signaling pathway. In contrast, PAI-1 mRNA and protein are not affected by TGF-β1 treatment in SKOV-3 cells. We have been studying the differences in invasive properties between the two cell lines. In agreement with Rodriguez et al. [2001], we found that HRA and SKOV-3 ovarian cancer cells remained sensitive to TGF-β-stimulated invasion through up-regulation of uPA expression. The in vitro invasion assay revealed that HRA cells markedly invade through Matrigel in response to TGF-β1, whereas SKOv-3 cells invade weakly. Our published data [Tanaka et al., 2003] and the present results showed that uPA is necessary for cancer cell invasion (HRA and SKOV-3), that the combination of TGF-β1 and PP2, which activates PAI-1 expression in SKOV-3 cells and reduces uPA expression in two cell lines, results in decreased invasiveness of both cells, and that the strong response of HRA cells to the invasive effects of TGF-β1 may be due to concomitant up-regulation of uPA and PAI-1 expression. These studies will provide insights into the mechanisms underlying the correlation between the biological phenotype of invasion and the activation or inhibition of various signaling pathways. Although PAI-1 may be involved in neutralizing an excess amount of endogenous uPA, PAI-1 may also work as an enhancer of cell attachment to ECM components. These results question a simplistic view of PAI-1 as an inhibition of cancer cell invasion.

With respect to TGF-β/Smad signaling, PAI-1 production is dictated by nuclear accumulation of the activated Smad complex [Attisano and Wrana, 2000]. In addition, nuclear accumulation of Smads can be inhibited by Ras-activated ERK kinases [Attisano and Wrana, 2000]. In our experiments, however, pharmacological inhibition of MAPK failed to affect Smad activation. The data reported by Attisano and Wrana [2000] is in contrast to our results in which Smad activation is induced in response to TGF-β1 in cells pretreated with Src kinase inhibitor PP2. Our observations support the perspective that specifically targeting Src and Smads represent a valid approach to blocking uPA and PAI-1 expression, which may results in suppression of cell invasion and metastasis. The overall observation would need to be more rigorously developed through the use of dominant negative inhibitors of Src and/or Smads.

The induction of PAI-1 mRNA and protein by TGF-β has been observed in several types of cultured cells [Westerhausen et al., 1991]. Cooperation between Smad2– or Smad3–Smad4 complexes and other sequence-specific DNA-binding proteins has been demonstrated to regulate TGF-β-responsive promoters [Chen et al., 1997; Labbé et al., 1998; Zhou et al., 1998]. The induction of PAI-1 protein was partially inhibited by mithramycin in SKOV-3 cells. The lack of complete inhibition is likely because PAI-1 promoter is induced by TGF-β not only through Sp1 sites in the proximal region (our data) but also through the distal region where Smads can bind to the CAGA box elements directly [Derynck and Feng, 1997; Dennler et al., 1998; Hua et al., 1998].

TGF-β/Smad signaling pathway is not functional in SKOV-3 cells. What factors can influence TGF-β resistance in SKOV-3 cells? Our previous data support a role for TGF-β1 activation of two distinct pathways (Src-MAPK-PI3K-NF-κB-dependent and Src-MAPK-AP-1-dependent) for TGF-β1-dependent uPA up-regulation and promotion of invasion [Kobayashi et al., 2003]. The principal finding of this study is that, in HRA cells, TGF-β1 also activate Smad2 phosphorylation and nuclear translocation of Smad3, leading to PAI-1 expression. This induction is unaffected by the Src inhibitor PP2, suggesting that it is independent of Src. Involvement of Src in invasiveness and metastatic development has been established in many neoplastic cells including HRA cells [Reddy et al., 2003]. On the other hand, in SKOV-3 cells, TGF-β1-mediated Src activation may directly or indirectly suppress the subsequent activation of Smads (Fig. 10). If abnormalities exist in the cellular response of TGF-β1 signals in SKOV-3 cells, they must lie upstream of the Smad proteins.

Increased levels of Smad3 or Smad4 can induce apoptosis [Yoo et al., 2003]. In the present

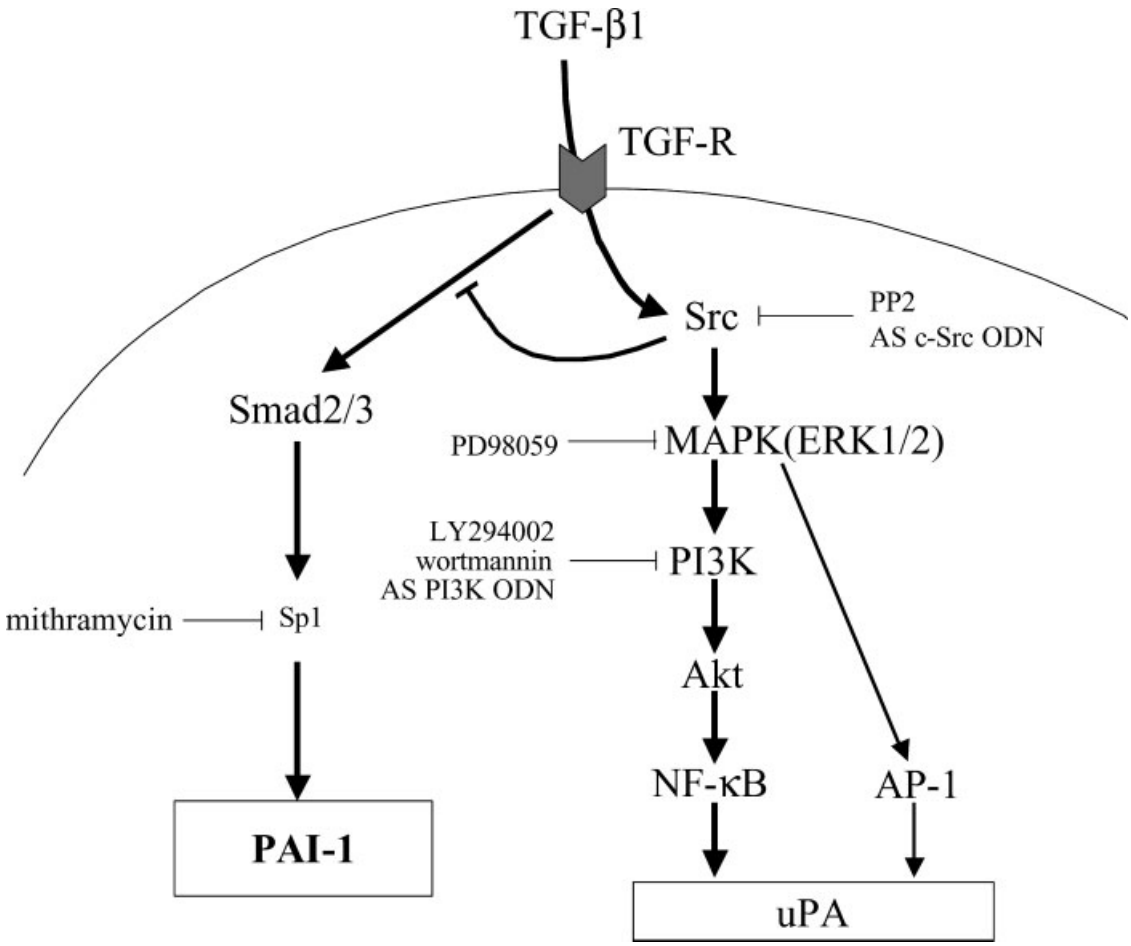

Fig. 10. Schematic representation of the TGF-β1-induced Src and Smad pathways involving uPA and PAI-1 synthesis. TGF-β1-induced Src pathway involving uPA expression is reviewed in Tanaka et al. [2004].

study, however, TGF-β1 neither induced apoptosis nor cell growth inhibition in ovarian cancer cells (data not shown). One possibility is that the Smad pathway may not be involved in TGF-β1-dependent signaling on apoptosis and growth in ovarian cancer cells.

In summary, we describe a novel pathway activated by TGF-β1: Src kinase appears to be the initial event of this pathway. HRA ovarian cancer cells are invasive in a TGF-β1 responsive way; this cytokine induces both uPA and PAI-1 expression. SKOV-3 cells are less invasive and TGF-β1 insensitive (PAI-1 is not induced by this cytokine). We demonstrate that Src inhibitors such as PP2 and AS c-Src probes can make the SKOV-3 sensitive to TGF-β1, modulating the Smad2/3 pathway and triggering PAI-1 expression. This is evidence that Src serves to negatively regulate PAI-1 expression via the Smad2/3 pathway. This in turn would suggest that the difference between the HRA and SKOV-3 lines lies in the ability to TGF-β1 to activate Src, something that is not addressed. The results obtained in the present reports reflect the complex biological effects of the PA system. Through involvement in TGF-β1 signaling and regulation of uPA and PAI-1 expression, Src protein tyrosine kinases and Smad proteins may serve as excellent therapeutic targets in the treatment of ovarian cancer.

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