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The steroid receptor co-activator-1 (SRC-1) potentiates TGF-β/Smad signaling: role of p300/CBP

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The three related 160-kDa proteins, SRC-1, TIF-2 and RAC-3, were initially identified as factors interacting with nuclear receptors. They have also been reported to potentiate the activity of other transcription factors such as AP-1 or NF-κB. The aim of this work was to identify whether SRC-1 interferes with the TGF-β/Smad signaling pathway, and if so, to identify its underlying mechanisms of action. Using transient cell transfection experiments performed in human dermal fibroblasts with the Smad3/4-specific $(SBE)_4$-lux reporter construct, as well as the human *PAI-1* promoter, we determined that SRC-1 enhances TGF-β-induced, Smad-mediated, transcription. Likewise, SRC-1 overexpression potentiated TGF-β-induced upregulation of *PAI-1* steady-state mRNA levels. Using a mammalian two-hybrid system, we demonstrated that SRC-1 interacts with the transcriptional co-activators p300/CBP, but not with Smad3. Overexpression of the adenovirus E1A oncoprotein, an inhibitor of CBP/p300 activity, prevented the enhancing effect of SRC-1 on Smad3/4-mediated transcription, indicating that p300/CBP may be required for SRC-1 effect. Such hypothesis was validated, as expression of a mutant form of SRC-1 lacking the CBP/p300-binding site failed to upregulate Smad3/4-dependent transcription, while full-length SRC-1 potentiated p300 · Smad3 interactions. These results identify SRC-1 as a novel Smad3/4 transcriptional partner, facilitating the functional link between Smad3 and p300/CBP.

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Keywords: p300; Smad; SRC-1 TGF-β

Introduction

Transforming growth factor-β (TGF-β) regulates a variety of cellular responses, including proliferation, differentiation, apoptosis and migration (Derynck and Zhang, 2003; Shi and Massagué, 2003). TGF-β elicits its cellular effects by inducing a heteromeric complex of two serine/threonine kinase receptors, the type I and type II receptors (TβRI and TβRII, Massagué and Weis-Garcia, 1996). Upon ligand binding, the constitutively active TβRII phosphorylates TβRI, allowing for intracellular signal propagation. Once activated, TβRI transiently associates with, and phosphorylates receptor-activated Smads (R-Smads) at their two carboxy-terminal serine residues (Attisano and Wrana, 2002; Derynck and Zhang, 2003; Shi and Massagué, 2003). R-Smads consist of two highly conserved Mad homology domains, termed MH1 and MH2, connected by a linker region (Shi *et al*., 1997). The MH1 domain is involved in DNA binding, while the MH2 domain is important for protein · protein interactions. In the basal state, R-Smads are kept in the cytoplasm bound to the protein SARA (Smad Anchor for Receptor Activation). Upon phosphorylation by activated TβRI, they are released from SARA and form heteromeric complexes with Smad4, a common mediator for all Smad pathways. These complexes are then translocated into the nucleus where they function as transcriptional regulators (Derynck and Zhang, 2003; Shi and Massagué, 2003). Members of the inhibitory Smad subclass, Smad6 and Smad7, bind activated TβRI and prevent phosphorylation and nuclear translocation of R-Smads, and recruit E3-type ubiquitin ligases to the receptors complexes, ultimately leading to their degradation (Derynck and Zhang, 2003; Shi and Massagué, 2003).

Several studies have demonstrated that the mechanism of Smad-mediated transcriptional activation involves the p300/CREB-binding protein (CBP) transcriptional co-activators. Both the receptor-activated Smad2 and Smad3 (Feng *et al*., 1998; Janknecht *et al*., 1998; Topper *et al*., 1998), and Smad4 (de Caestecker *et al*., 2000) bind directly to p300/CBP, and Smad-mediated transcription is dependent on the co-activator function of p300/CBP. The latter proteins modify transcription either by altering chromatin structure so that the underlying DNA sequences are exposed to the transcriptional apparatus (Workman and Kingston, 1998) or by directly recruiting the RNA polymerase II holo-enzyme to the promoter (Snowden and Perkins, 1998).

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Three related 160-kDa proteins, SRC-1, TIF-2 and RAC-3, encoded by separate genes, form the steroid receptor co-activator or p160 family of co-activators (McKenna *et al*., 1999; Glass and Rosenfeld, 2000). These proteins are highly homologous and were initially identified as factors that interact with nuclear receptors (NRs) and enhance NR-dependent transcriptional activation (Onate *et al*., 1995; Voegel *et al*., 1996; Li *et al*., 1997). p160 proteins have also been reported to potentiate the activity of several other transcription factors, including AP-1 and NF-κB (Kim *et al*., 1998; Lee *et al*., 1998, 1999; Na *et al*., 1998; Carrero *et al*., 2000), although the mechanisms by which p160 proteins enhance the various transcription factor activities still remains to be characterized in greater detail.

In the present study, we provide evidence that SRC-1 enhances the functional link between Smad3 and p300/CBP, resulting in the enhancement of TGF-β-induced, Smad3-mediated, transcription.

Results

SRC-1 enhances TGF-β/Smad-mediated transcription

To determine the effect of SRC-1 on TGF-β-induced Smad3/4-mediated, transcriptional response, human dermal fibroblasts were transfected with a full-length SRC-1 expression vector together with the synthetic SBE-driven reporter plasmid termed $(SBE)_4$-lux, containing four Smad3 and Smad4 binding elements. As shown in Figure 1a, overexpression of SRC-1 enhances TGF-β-driven Smad3/4-specific promoter transactivation in a dose-dependent manner.

To examine the physiological relevance of our findings, the modulation of the *PAI-1* steady-state mRNA levels by TGF-β was specifically measured by Northern hybridization of RNA extracted from cells transfected or not with an SRC-1 expression vector (Figure 1b). Strong enhancement of TGF-β-driven elevation of *PAI-1* mRNA steady-state levels was observed in response to SRC-1 (Figure 1b, left panel, lane 4 *vs* 2). Quantitation of the autoradiograms and correction of stimulation activity of SRC-1 on TGF-β-induced *PAI-1* transcription values against those obtained for *GAPDH* in the same samples indicated a 3.8-fold elevation of *PAI-1* mRNA levels (Figure 1b, right panel). Of note, SRC-1 overexpression alone did not affect basal *PAI-1* expression level (Figure 1b, lane 3 *vs* lane 1).

To determine whether the effect of SRC-1 on PAI-1 expression was transcriptional, we tested the effect of SRC-1 on *PAI-1* promoter transactivation. As shown in Figure 1c (left panel), SRC-1 dramatically potentiated TGF-β-induced upregulation of p800-Lux, a reporter construct that contains 800 bp of the *PAI-1* promoter, a

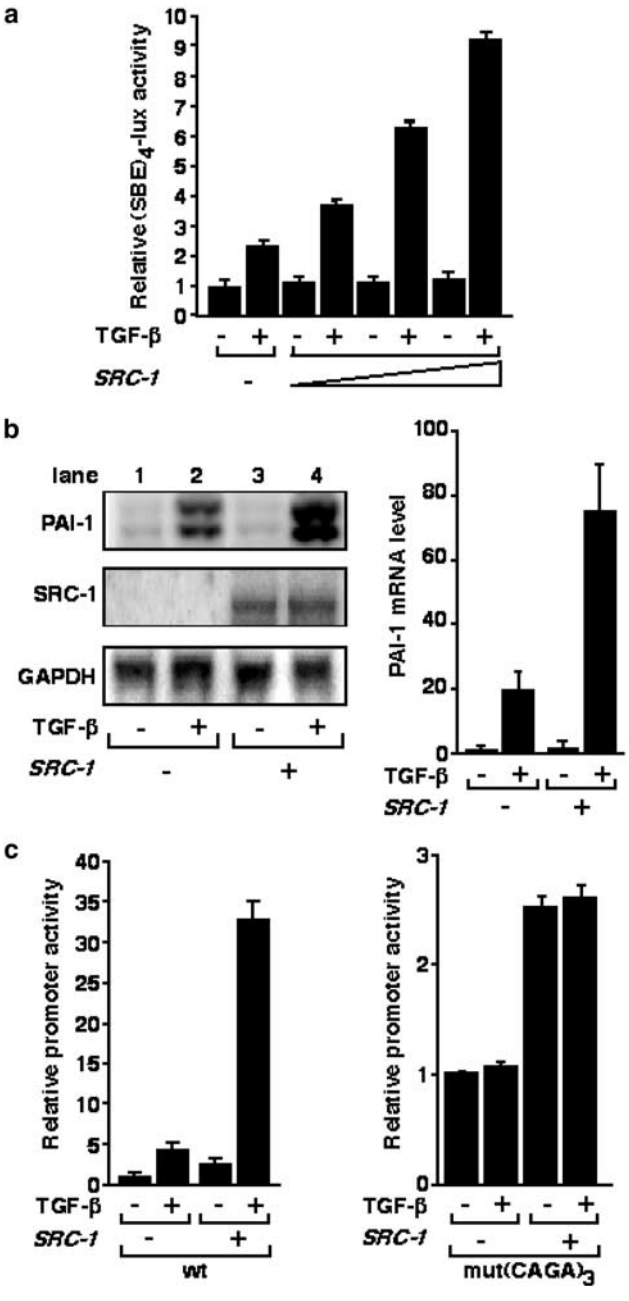

Figure 1 SRC-1 stimulates TGF-β-driven Smad3/4-specific transcriptional activity. Subconfluent fibroblast cultures were transfected with $(SBE)_4$-lux and increasing amounts of SRC-1 expression vector (**a**). After 3 h, TGF-β (10 ng/ml) was added, and incubations continued for 24 h before luciferase assays. Bars indicate mean ± s.d. of at least three independent experiments performed each with duplicate samples. (**b**) Subconfluent fibroblast cultures transfected with either an empty (−) or SRC-1 (+) expression vector were treated with TGF-β (10 ng/ml) for 24 h in medium containing 1% serum. After incubations, *PAI-1* (upper panel) and *SRC-1* (middle panel) mRNA levels was detected by Northern blot analysis. Specificity of the modulation was confirmed using a specific *GAPDH* probe (lower panel). Quantitation of the autoradiograms: *PAI-1* mRNA levels are corrected against those for *GAPDH* in the same samples. (**c**) Subconfluent fibroblast cultures were transfected with p800-lux (left panel) or mut$(CAGA)_3$ (right panel) in the absence or presence of SRC-1 expression vector. After 3 h, TGF-β (10 ng/ml) was added, and incubations continued for 24 h before luciferase assays. Bars indicate mean ± s.d. of three independent experiments performed each with duplicate samples

prototypic TGF-β/Smad3/4 target (Dennler *et al*., 1998). To determine whether the effect of SRC-1 was dependent on Smad3 specifically, we repeated the experiments of transient cell transfections with a *PAI-1* promoter fragment in which the three Smad3/4-response elements (CAGA boxes) allowing TGF-β response have been rendered nonfunctional simultaneously (Dennler *et al*., 1998). The results of these experiments demonstrated that the mut$(CAGA)_3$ construct, no longer responsive to TGF-β, does not permit any enhancing activity of SRC-1 in the presence of TGF-β (Figure 1c, right panel).

To further investigate the implication of Smad3 and Smad4 on SRC-1 ability to enhance TGF-β-driven transcription, human dermal fibroblasts were co-transfected with SRC-1, Smad3 and Smad4 expression vectors together with $(SBE)_4$-lux or p800-lux reporter constructs. As shown in Figure 2a and b, simultaneous transfection of Smad3 or Smad4 together with SRC-1 dramatically increased TGF-β-driven $(SBE)_4$-lux and p800-lux transactivation, as compared to transfections with Smad3 and/or Smad4 expression vectors, without SRC-1.

Next, cells were transfected with expression vectors encoding either a dominant-negative form for Smad3 (D/N Smad3) lacking the N-terminal MH2 domain or the inhibitory Smad, Smad7, together with $(SBE)_4$-lux or p800-lux constructs. As shown in Figures 2c and d, in the presence of either D/N Smad3 or Smad7, SRC-1 failed to enhance TGF-β-driven Smad3/4-specific promoter transactivation.

From these experiments, we conclude that SRC-1 potentiates TGF-β effect on *PAI-1* gene expression specifically at the level of Smad3/4-dependent transcription.

SRC-1 is a co-activator for Smad3

R-Smads exhibit intrinsic transcriptional activity when fused to the Gal4 DNA-binding domain (Liu *et al*., 1996). In order to determine if SRC-1 protein could directly affect the transcriptional activity of Smad3, cells were transfected with a luciferase reporter gene containing five Gal4-binding sites upstream of a minimal promoter and Gal4BD-Smad3 and SRC-1 expression vectors. Following transfection, cells were grown in the presence or absence of TGF-β. As shown in Figure 3a, expression of SRC-1 enhanced the transcriptional activity of Gal4BD-Smad3 upon TGF-β stimulation. SRC-1 alone had no effect on the luciferase activity when cells were transfected with only the Gal4 DNA-binding domain. These results demonstrate the co-activator function of SRC-1 for Smad3.

To test the possibility that SRC-1 protein also affects the ability of Smad3 to bind its cognate DNA binding motif (SBE), we performed EMSA using nuclear

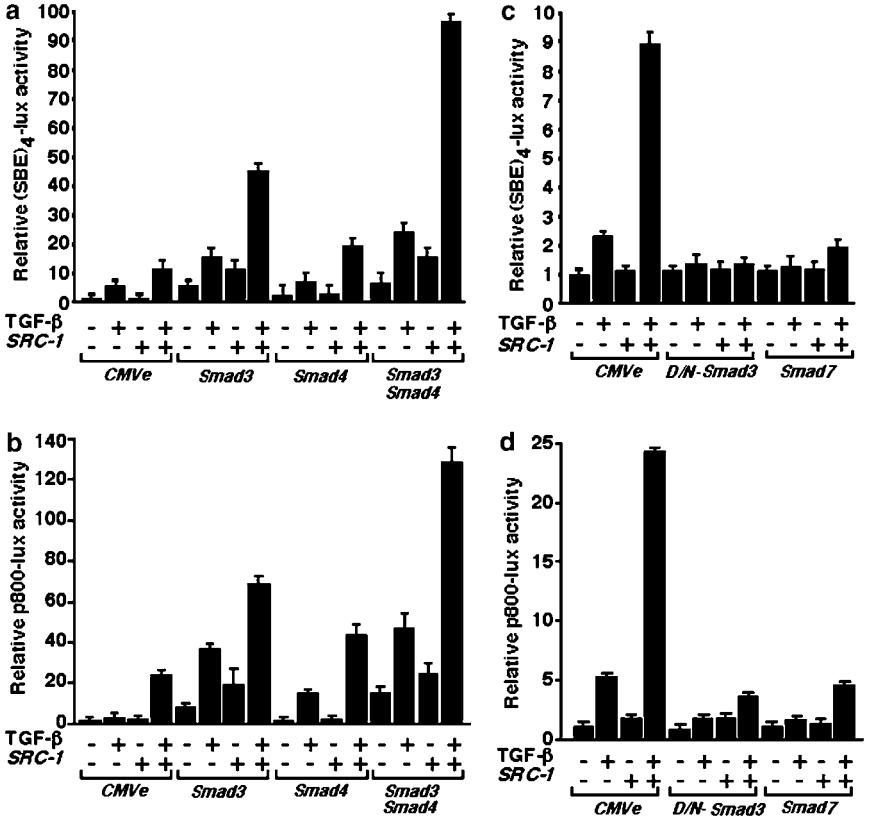

Figure 2 SRC-1 enhances Smad3-induced $(SBE)_4$-lux and p800-lux transcriptional activity. Subconfluent fibroblast cultures were co-transfected with $(SBE)_4$-lux (**a** and **c**) or p800-lux (**b** and **d**), and the indicated combinations of expression vectors for Smad3, Smad4, D/N Smad3 or Smad7. After 3 h, TGF-β (10 ng/ml) was added as indicated, and incubations continued for 24 h before luciferase assays were performed. Bars indicate mean $\pm$ s.d. of at least three independent experiments performed each with duplicate samples

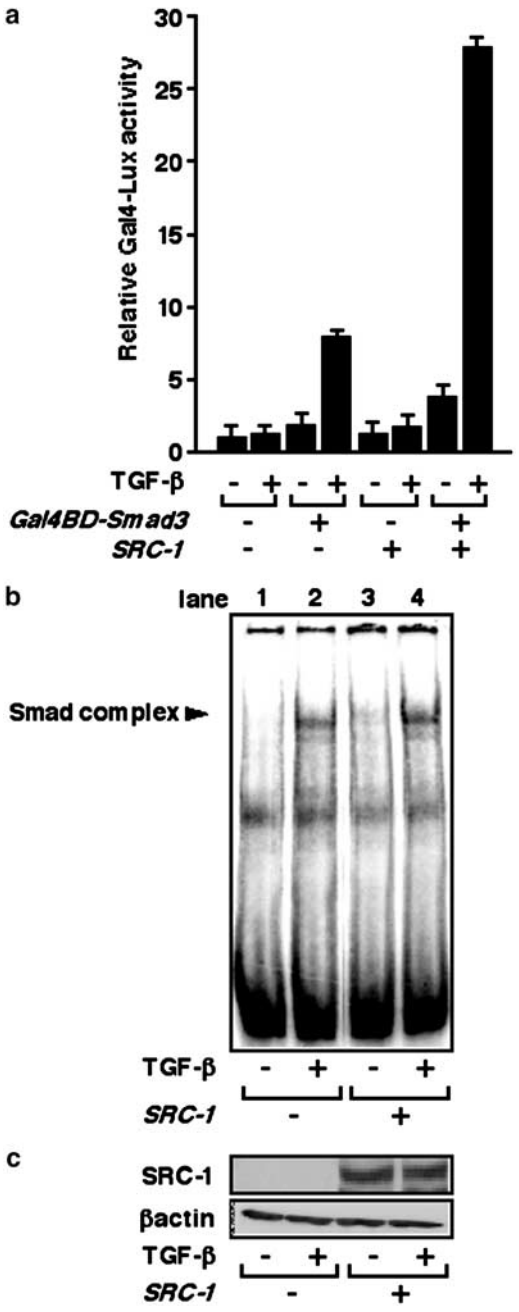

Figure 3 SRC-1 potentiates the transcriptional activity of Gal4-Smad3 without modifying the affinity of Smad3 for its cognate SBE binding motif. (**a**) Fibroblast cultures were co-transfected with Gal4-lux together with the indicated combinations of SRC-1 and either Gal4BD or Gal4BD-Smad3 expression vectors, without or with TGF-β (10 ng/ml). Bars indicate mean $\pm$ s.d. of at least three independent experiments performed each with duplicate samples. (**b**) Gel shift assays were performed using a Smad3/4-specific $3\times$CAGA oligonucleotide as a probe and nuclear extracts from fibroblasts transfected with the SRC-1 expression vector and treated for 30 min with TGF-β (10 ng/ml). (**c**) The expression of SRC-1 was measured by applying one-fiftieth of the total lysate on SDS–PAGE followed by Western blotting with HA and β-actin antibodies

extracts from fibroblast cultures transfected or not with SRC-1 expression vector, treated or not with TGF-β. As expected, TGF-β induced a single specific shifted band (Figure 3b, lane 2). The latter was not modified when SRC-1 was overexpressed (lane 4). The presence of identical levels of SRC-1 protein in all samples was verified by Western blotting (Figure 3c).

Together, these results suggest that SRC-1 stimulates the intrinsic transcriptional activity of Smad3 without modifying the affinity of Smad3/4 complexes for their cognate DNA binding motif.

SRC-1 interacts with p300, not with Smad3

Recent studies have shown that TGF-β/Smad3-mediated transcriptional activation involves the p300/CBP transcriptional co-activators (Feng *et al*., 1998; Janknecht *et al*., 1998; Topper *et al*., 1998; de Caestecker *et al*., 2000). To understand the mechanism of SRC-1 action, it was therefore important to determine whether SRC-1 functions as a Smad co-activator in a p300/CBP-dependent or -independent manner. To this aim, we first examined the possibility that SRC-1 and Smad3 proteins may undergo direct protein$\cdot$protein interactions. In the mammalian two-hybrid, Gal4-based, transactivation assay, we demonstrated that VP16AD-SRC-1 does not enhance Gal4BD-Smad3-mediated transcription, neither in the absence nor in the presence of TGF-β (Figure 4a), suggesting that Smad3 does not directly interact with SRC-1, as previously evoked by Yanagisawa *et al*. (1999). In contrast, the physical interaction between SRC-1 and p300, previously described in the literature (Glass and Rosenfeld, 2000), was confirmed in another mammalian two-hybrid assay, as expression of VP16AD-SRC-1 potently enhanced Gal4BD-p300-mediated transactivation (Figure 4b), reflecting direct SRC-1/p300 interaction.

Enhancement of Smad3/4 mediated transcription by SRC-1 requires functional p300 co-activators

As a first attempt to address whether p300/CBP are functionally required for the enhancement of Smad-mediated transcription by SRC-1, we evaluated the effect of the adenovirus E1A oncoprotein, an inhibitor of CBP/p300 activity (Arany *et al*., 1995), on the ability of SRC-1 to enhance Smad3/4 transcriptional activity. As shown in Figure 5a and b, E1A overexpression reduced both TGF-β-driven $(SBE)_4$-lux and p800-lux promoter transactivation, and the effect of SRC-1 on TGF-β-induced Smad3/4 specific promoter transactivation, while a mutant form of E1A lacking the CBP/p300-binding site (Kraus *et al*., 1992) had no effect on these responses. Next, to confirm that SRC-1 effect on Smad3/4 transcriptional activity was dependent on p300 activity, we co-transfected increasing amounts of p300 with E1A expression vectors. As shown in Figure 5c and d, p300 relieved E1A inhibition of SRC-1 effect on TGF-β-driven Smad3/4 specific promoter transactivation. To ascertain the role played by p300, we then tested the effect of a mutant form of SRC-1 laking the

CBP/p300-binding site (Chauchereau *et al*., 2000) on Smad-dependent transcription. As shown in Figure 6a and b, expression of the truncated SRC-1 failed to enhanced TGF-β-driven $(SBE)_4$-lux and p800-lux promoter transactivation, confirming the need for p300 in SRC-1-mediated enhancement of Smad3/4-dependent transcription.

To determine whether SRC-1 activity may play a role in controlling Smad3 · p300 interactions, we adapted the mammalian two-hybrid, Gal4-based, transactivation assay. As shown in Figure 6c, in the absence of exogenous SRC-1, significant transactivation of Gal4-lux was observed when cells were transfected with Gal4BD-p300 and VP16AD-Smad3 and treated with TGF-β. In the presence of SRC-1 expression vector, expression of VP16AD-Smad3 dramatically enhanced Gal4BD-p300-mediated transactivation, which was further enhanced by TGF-β, representative of strong interactions between Smad3 and p300. On the other hand, overexpression of a mutant form of SRC-1 lacking the CBP/p300-association domain had no effect on Smad3 · p300 interaction, neither in the absence nor in the presence of TGF-β.

Taken together, these results provide strong evidence for a role of SRC-1 in enhancing Smad3 · p300 direct interactions.

To further define the role of p300 in the ability of SRC-1 to enhance TGF-β/Smad3/4-driven transcriptional activity, cells were transfected with SRC-1 and p300 expression vectors together with $(SBE)_4$-lux reporter construct. As shown in Figure 7a, p300 co-expression enhances TGF-β-driven $(SBE)_4$-lux transactivation, and p300 dramatically increases SRC-1 ability to enhance TGF-β-driven Smad3/4 specific promoter transactivation. Similar results were obtained with the p800-lux reporter construct (Figure 7b).

To examine the relevance of our findings on the regulation of an endogenous Smad3 target gene, we examined the modulation of *PAI-1* steady-state mRNA levels upon TGF-β stimulation by Northern analysis of RNA extracted from fibroblasts transfected or not with a combination of SRC-1 and p300 expression vectors. In agreement with the data obtained at the promoter level, TGF-β treatment resulted in a strong elevation of *PAI-1* mRNA steady-state levels (Figure 7c, lane 2 *vs* lane 1). Visual examination of the autoradiograms suggested that TGF-β effect was potentiated by both p300 (lane 4 *vs* lane 2) and SRC-1 (lane 6 *vs* lane 2), the two acting in concert to further enhance TGF-β response (lane 8 *vs* lane 2). Quantitation of the autoradiograms and correction of *PAI-1* levels against variations of *GAPDH* levels in the same samples (Figure 7d) confirmed that TGF-β response (about 20-fold elevation of *PAI-1* expression levels as compared to unstimulated fibroblasts) was enhanced 3.8- and 3.7-fold in the presence of SRC-1 and p300, respectively. Furthermore, concomitant expression of SRC-1 and p300, while not affecting

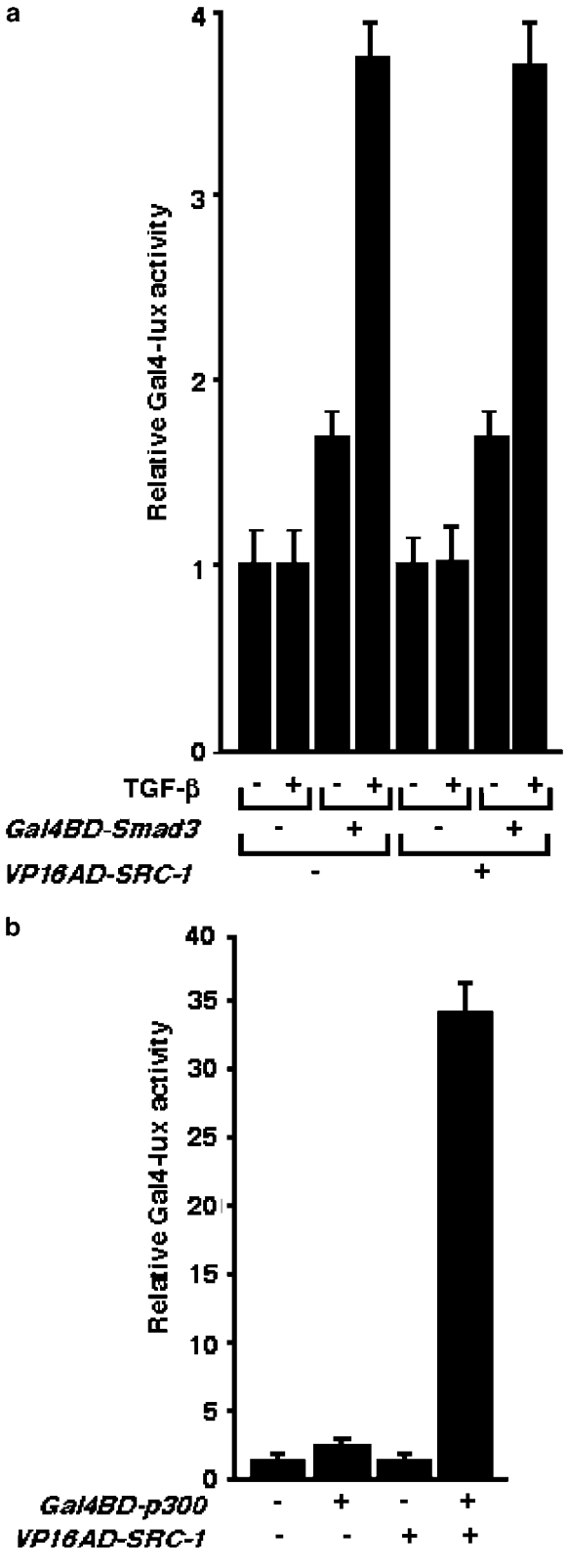

Figure 4 SRC-1 interacts with p300 but not with Smad3. Subconfluent fibroblast cultures were co-transfected with Gal4-lux and VP16AD-SRC-1 together with Gal4BD-Smad3 (**a**) or Gal4BD-p300 (**b**) expression vectors. Where indicated (**a**), TGF-β (10 ng/ml) was added 3 h after transfections, and incubations continued for 24 h before luciferase assays. Bars indicate mean ± s.d. of at least three independent experiments performed each with duplicate samples

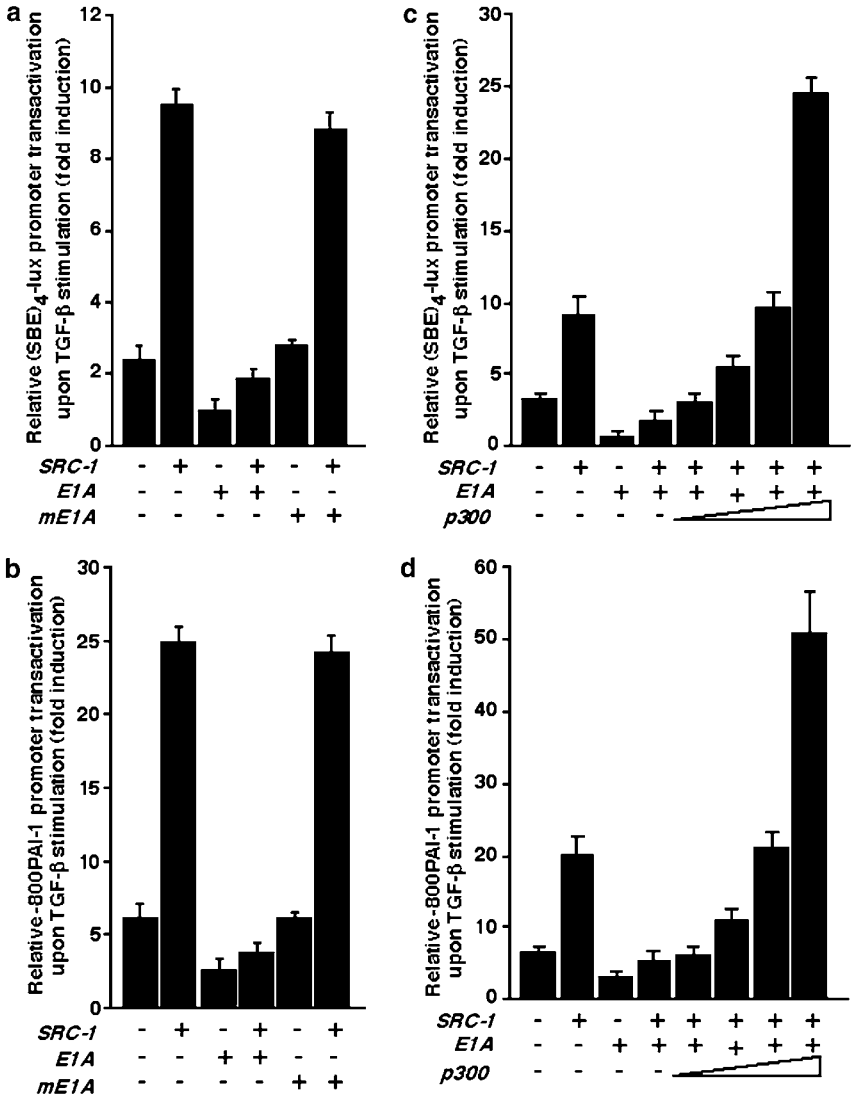

Figure 5 Enhancement of Smad3/4-mediated transcription by SRC-1 requires functionally active p300/CBP. Subconfluent fibroblast cultures were transfected with $(SBE)_4$-lux (**a** and **c**) or p800-lux (**b** and **d**) together with the indicated combinations of expression vectors for SRC-1, E1A and mE1A, (**a** and **b**) or with increasing amounts of p300 (**c** and **d**). At 3 h after transfection, cells were treated with TGF-β (10 ng/ml) for 24 h before reporter gene activity was assayed. Results are shown as fold-activation of TGF-β effect on promoter activity. Bars indicate mean $\pm$ s.d. of at least three independent experiments performed each with duplicate samples

basal *PAI-1* mRNA expression levels, resulted in a 12-fold enhancement of the TGF-β response.

Taken together, these results suggest a functional cooperation between p300 and SRC-1 to stimulate TGF-β-induced, Smad3/4-dependent, transcriptional activity.

Discussion

Previous studies have shown that transcriptional activation by Smad3 occurs, in large part, by its ability to recruit the general co-activator p300 (Feng *et al*., 1998; Janknecht *et al*., 1998; Pouponnot *et al*., 1998; Shen *et al*., 1998). In addition, some of the observed cooperative effects of Smads and other DNA-binding proteins may be at the level of recruitment of other co-activators. For example, the vitamin D receptor (VDR) has been shown to physically and functionally interact with Smad3 resulting in Smad3-enhanced transactivation function of VDR (Yanagisawa *et al*., 1999). In their study, the authors demonstrated that the interaction between VDR and Smad3 requires at least a member of the SRC-1 protein family.

In this report, we demonstrated that SRC-1 is able to enhance TGF-β-induced, Smad3/4-mediated, transcription. We also demonstrated that SRC-1 does not directly interact with Smad3 as previously suggested (Yanagisawa *et al*., 1999) but interacts directly with the transcriptional co-activators p300/CBP, suggesting that p300/CBP could play a crucial role in SRC-1-enhancing Smad3-mediated transcriptional activity upon TGF-β stimulation. Overexpression of the adenovirus E1A

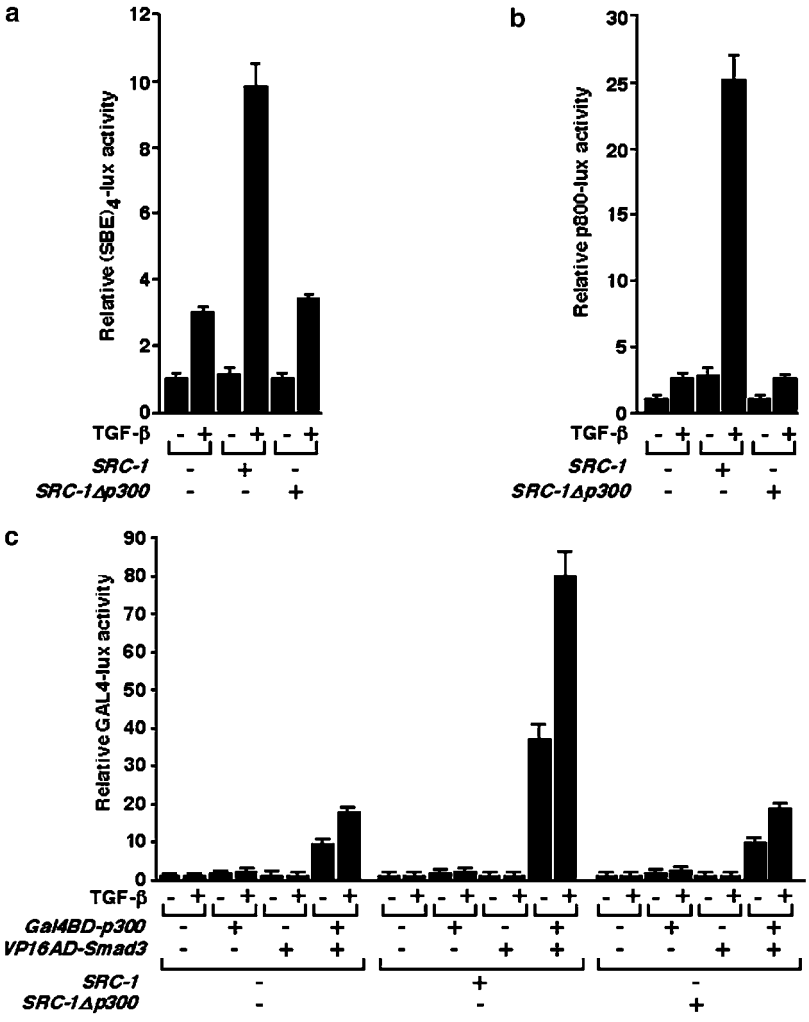

Figure 6 A truncated form of SRC-1 lacking the CBP/p300 interacting domain fails to enhance Smad3/4-driven transcriptional activity upon TGF-β stimulation and fails to potentiate p300 · Smad3 interactions. Subconfluent fibroblast cultures were co-transfected with $(SBE)_4$-lux (**a**) or p800-lux (**b**) together with the indicated combinations of expression vectors for SRC-1 or SRC-1Δp300. At 3 h after transfections, cells were treated with TGF-β (10 ng/ml) for 24 h before reporter gene activity was assayed. Bars indicate mean ± s.d. of at least three independent experiments performed each with duplicate samples. (**c**) Fibroblasts were co-transfected with Gal4-lux together with expression vectors for Gal4BD-p300, VP16AD-Smad3, SRC-1 or SRC-1Δp300, as indicated. At 3 h after transfections, TGF-β (10 ng/ml) was added, and incubations continued for 24 h before luciferase activity was determined. Bars indicate mean ± s.d. of at least 3 independent experiments performed each with duplicate samples

oncoprotein, an inhibitor of CBP/p300 activity, prevented the enhancing effect of SRC-1 on Smad3/4-mediated transcription, further suggesting that p300/CBP may be required for SRC-1 effect. Indeed, overexpression of a mutant form of SRC-1 lacking the CBP/p300-binding site failed to upregulate Smad3/4-dependent transcription. In addition, we found that SRC-1 potentiates p300.Smad3 interactions while not binding Smad3 directly. Together, these results identify SRC-1 as a novel Smad3/4 transcriptional partner facilitating the functional link between Smad3 and p300/CBP, resulting in the enhancement of TGF-β-mediated transcription. Our results suggest that SRC-1 could play a crucial role for the crosstalk between TGF-β and vitamin D signaling pathway enhancing both transactivation functions of Smad3 (our work) and VDR (Yanagisawa *et al*., 1999).

Nuclear receptors regulate target gene expression in response to steroid and thyroid hormones, retinoids, vitamin D and other ligands. These ligand-dependent transcription factors function by contacting various nuclear cooperating proteins, called co-activators and co-repressors, which mediate local chromatin remodeling as well as communication with the basal transcriptional apparatus. Nuclear receptors and their co-regulatory proteins play a role in cancer. Co-regulators are often present in limiting amounts in cell nuclei and modifications of their expression level and/or structure lead to alterations in nuclear receptor functioning, which may be as pronounced as a complete inversion of signaling, that is, from stimulating to repressing certain genes in response to an identical stimulus. In addition, hemizygous knockout of certain co-activator genes have been demonstrated to produce cancer-prone

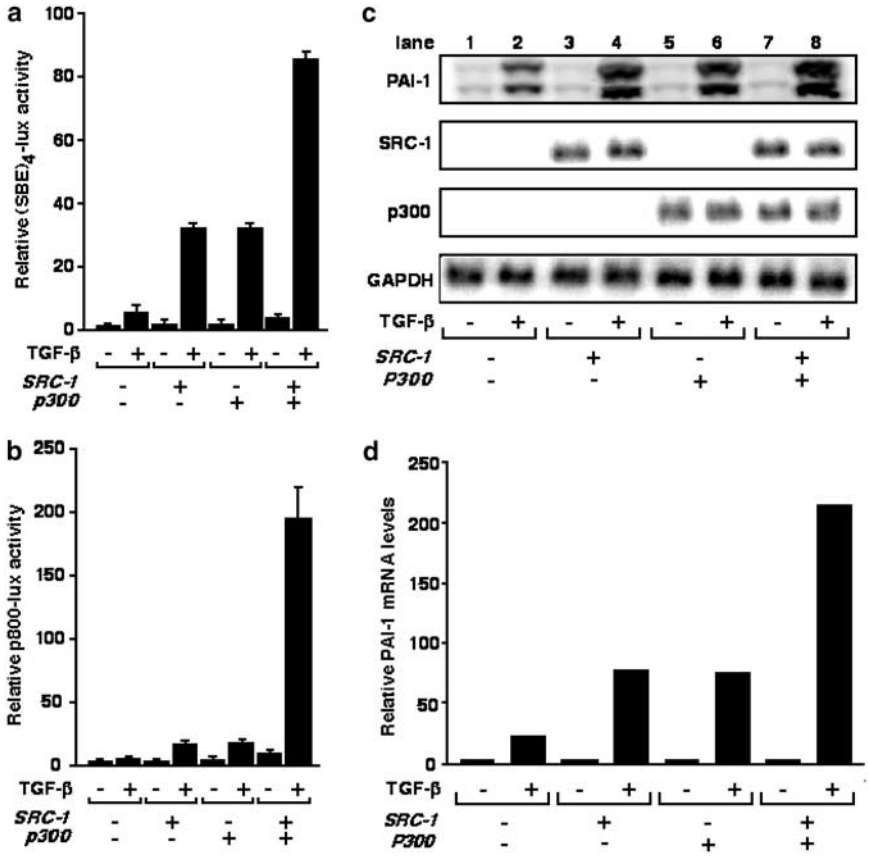

Figure 7 p300 and SRC-1 synergize to stimulate TGF-β-driven Smad3/4-specific promoter transcriptional activity and to enhance TGF-β-mediated elevation of endogenous PAI-1 mRNA levels. Subconfluent fibroblast cultures were co-transfected with $(SBE)_4$-lux (**a**) or p800-lux (**b**) together with expression vectors for SRC-1 and p300, as indicated. At 3 h after transfection, cells were treated with TGF-β (10 ng/ml) for 24 h before reporter gene activity was determined. Bars indicate mean $\pm$ s.d. of at least three independent experiments performed and each with duplicate samples. (**c**) Subconfluent fibroblast cultures, transfected by electroporation with a combination of SRC-1 and/or p300 expression vectors, were treated with TGF-β (10 ng/ml) for 24 h, then *PAI-1*, *SRC-1* and *p300* mRNA levels were detected by Northern analysis. Specificity of the modulation was confirmed using a specific *GAPDH* probe. (**d**) Quantitation of *PAI-1* mRNA steady-state levels corrected against *GAPDH* expression values in the same samples

phenotypes in mice. Thus, assessment of co-activator and co-repressor expression and structure in tumors may turn out to be essential to determine the role of nuclear receptors in cancer and to predict prognosis and response to therapy (Cottone *et al*., 2001). For example, levels of SRC-1, in a study of 21 breast cancers, appeared to be able to predict case response to tamoxifen in patients with recurrent breast cancer (Berns *et al*., 1998). AIB1, a member of SRC-1 family, was found amplified in breast, ovarian, pancreatic, and gastric cancer (Guan *et al*., 1996; Anzick *et al*., 1997; Ghadimi *et al*., 1999; Sakakura *et al*., 2000). Amplification of the AIB1 gene was correlated with estrogen and progesterone receptor expression by primary breast tumors as well as with tumor size (Bautista *et al*., 1998). Independent of any amplification event, increased expression levels of AIB1 also have been found in primary breast tumors (Guan *et al*., 1996; Bouras *et al*., 2001; List *et al*., 2001).

In contrast to the tumor suppressor activity of TGF-β, tumor cells often show increased production of this growth factor, and considerable evidence documents its tumor-promoting role through its effects on tumor cell invasion and changes in the tumor microenvironment. For example, during the early stages of breast cancer development, the transformed epithelial cells appear to still be sensitive to TGF-β-mediated growth arrest, and TGF-β can act as an antitumor promoter. In contrast, advanced breast cancers are mostly refractory to TGF-β-mediated growth inhibition and produce large amounts of TGF-β, which may enhance tumor cell invasion and metastasis by its effects on extracellular matrix. Tumor metastasis depends on various factors, including the ability of tumor cells to migrate and invade the stroma, and to migrate in and out of blood and lymphatic vessels. The epithelial to mesenchymal differentiation of tumor cells has an important role in this invasive phenotype (for reviews, see Derynck *et al*., 2001; Wakefield and Roberts, 2002).

Gene regulation is a complex process that involves the coordinated integration of distinct different signal transduction pathways. Thus, our results suggest that SRC-1 may be a link between TGF-β and steroid signaling pathways particularly in enhancing tumor

progression in advanced cancer. Such hypothesis may prove especially important in the context of prostate cancer as SRC-1 and other co-activators including CBP are overexpressed in the luminal epithelial cells of the prostate, where over 90% of prostate tumors arise. Since there is growing evidence that nuclear receptor cofactors may be implicated in the progression of tumors, novel targets against tumor progression could include SRC-1 (Powell *et al*., 2004).

Materials and methods

Cell cultures

Human dermal fibroblasts were established by explanting neonatal foreskins. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, and antibiotics (100 U/ml penicillin, 50 μg/ml streptomycin-G and 0.25 μg/ml Fungizone™). Cell cultures were placed in medium containing 1% serum 24 h prior to experiments. Human recombinant TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). It is referred to as TGF-β throughout the text.

Plasmid constructs

$(SBE)_4$-lux, a Smad3/4-specific reporter construct (Zawel *et al*., 1998), was a kind gift from Dr Scott Kern (Johns Hopkins University, Baltimore, MD, USA). To measure prototypic TGF-β responses in the context of natural promoter sequences identified as Smad3 targets, we used p800-lux (a gift from Dr David J Loskutoff, Scribbs Research Institute, San Diego, CA, USA) which contains 800 bp of human plasminogen activator inhibitor-1 (PAI-1) promoter (Keeton *et al*., 1991; Dennler *et al*., 1998) cloned upstream of the luciferase gene. Tagged Smad3, Smad4, and Smad7 expression vectors (gifts from Dr A Roberts, NIH, Bethesda, MD, USA) were previously described (de Caestecker *et al*., 1998). Plasmids encoding the wild-type human SRC-1 (pSG5-HA-SRC-1) and deletion mutant (pSG5-HA-Δp300SRC-1) (kind gift from E Milgrom, INSERM U135, Le Kremlin-Bicetre, France), p300 (kind gift from Dr T Shioda, Boston, MA, USA), E1A and deletion mutant mE1A (gift from Dr A Roberts, NIH, Bethesda, MD, USA) have been previously described (respectively by Chauchereau *et al*., 2000; de Caestecker *et al*., 2000).

Protein · protein interactions were determined using a Gal4-based mammalian two-hybrid system. G5E1b-Lux containing five Gal4 binding sites driving the expression of luciferase and Gal4BD-Smad3 fusion protein expression vector, containing the full-length Smad3 cDNA have been described previously (Atfi *et al*., 1997). Additional expression vectors used in this study were: Gal4BD-p300 (a kind gift from Dr A Giordano, Thomas Jefferson University, Philadelphia, PA, USA) (Yuan *et al*., 1996) and VP16AD-SRC-1 (kind gift from E Milgrom, INSERM U135, Kremlin-Bicetre, France) (Chauchereau *et al*., 2000).

Transient cell transfections and reporter assays

Transient cell transfections of human dermal fibroblasts were performed with the calcium phosphate/DNA co-precipitation procedure using a commercial assay kit (Promega Corp., Madison, WI, USA). pRSV-β-galactosidase was co-transfected in every experiment to monitor transfection efficiency. Luciferase activity was determined with a commercial kit (Promega). For high transfection efficiency of SRC-1 and p300 expression vectors, cells were electroporated with a Nucleofector™ (Amaxa GmbH, Koeln, Germany) according to the manufacturer's protocol. Electroporation efficiency was estimated by FACS analysis of a co-transfected GFP expression vector. Typically, 65–75% of cells were transfected, allowing for direct assessment, at the level of endogenous *PAI-1* gene regulation.

Northern blotting

Total RNA was obtained using an RNeasy™ kit (Qiagen GmbH, Hilden Germany), and analysed by Northern hybridization with ^{32}P-labeled cDNA probes for *PAI-1*, *p300*, *SRC-1* and *GAPDH*. Quantitation was performed with a phosphorimager (Amersham-Pharmacia).

Electrophoresis mobility shift assays

A 3 × CAGA Smad-specific oligonucleotide (Dennler *et al*., 1998) was used as a probe to determine Smad/DNA interactions. Nuclear extracts were isolated using a small-scale preparation (Andrews and Faller, 1991). Binding mixtures were separated electrophoretically on 4% acrylamide gels in 1 × Tris-Acetate-EDTA buffer, pH 8.0.

References

Andrews NC and Faller DV. (1991). *Nucleic Acids Res.*, **19,** 2499.

Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM and Meltzer PS. (1997). *Science*, **277,** 965–968.

Arany Z, Newsome D, Oldread E, Livingston DM and Eckner R. (1995). *Nature*, **374,** 81–84.

Atfi A, Buisine M, Mazars A and Gespach C. (1997). *J. Biol. Chem.*, **272,** 24731–24734.

Attisano L and Wrana JL. (2002). *Science*, **296,** 1646–1647.

Bautista S, Valles H, Walker RL, Anzick S, Zeillinger R, Meltzer P and Theillet C. (1998). *Clin. Cancer Res.*, **4,** 2925–2929.

Berns EM, van Staveren IL, Klijn JG and Foekens JA. (1998). *Breast Cancer Res. Treat.*, **48,** 87–92.

Bouras T, Southey MC and Venter DJ. (2001). *Cancer Res.*, **61,** 903–907.

Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H and Poellinger L. (2000). *Mol. Cell Biol.*, **20,** 402–415.

Chauchereau A, Georgiakaki M, Perrin-Wolff M, Milgrom E and Loosfelt HJ. (2000). *J. Biol. Chem.*, **275,** 8540–8548.

Cottone E, Orso F, Biglia N, Sismondi P and de Bortoli M. (2001). *Int. J. Biol. Markers*, **16,** 151–166.

deCaestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB and Lechleider RJ. (1998). *Genes Dev.*, **12,** 1587–1592.

de Caestecker MP, Yahata T, Wang D, Parks WT, Huang S, Hill CS, Shioda T, Roberts AB and Lechleider RJ. (2000). *J. Biol. Chem.*, **275,** 2115–2122.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S and Gauthier JM. (1998). *EMBO J.*, **17,** 3091–30100.

Derynck R, Akhurst RJ and Balmain A. (2001). *Nat. Genet.*, **29,** 117–129.

Derynck R and Zhang YE. (2003). *Nature*, **425,** 577–584.

Feng XH, Zhang Y, Wu RY and Derynck R. (1998). *Genes Dev.*, **12,** 2153–2163.
Ghadimi BM, Schrock E, Walker RL, Wangsa D, Jauho A, Meltzer PS and Ried T. (1999). *Am. J. Pathol.*, **154,** 525–536.
Glass CK and Rosenfeld MG. (2000). *Genes Dev.*, **14,** 121–141.
Guan XY, Xu J, Anzick SL, Zhang H, Trent JM and Meltzer PS. (1996). *Cancer Res.*, **56,** 3446–3450.
Janknecht R, Wells NJ and Hunter T. (1998). *Genes Dev.*, **12,** 2114–2119.
Keeton MR, Curriden SA, van Zonneveld AJ and Loskutoff DJ. (1991). *J. Biol. Chem.*, **266,** 23048–23052.
Kim HJ, Kim JH and Lee JW. (1998). *J. Biol. Chem.*, **273,** 28564–28567.
Kraus VB, Moram E and Neirns JR. (1992). *Mol. Cell Biol.*, **12,** 4391–4399.
Lee SK, Kim HJ, Na SY, Kim TS, Choi HS, Im SY and Lee JW. (1998). *J. Biol. Chem.*, **273,** 16651–16654.
Lee SK, Kim HJ, Kim JW and Lee JW. (1999). *Mol Endocrinol.*, **13,** 1924–1933.
Li H, Gomes PJ and Chen JD. (1997). *Proc. Natl. Acad. Sci. USA*, **94,** 8479–8484.
List HJ, Reiter R, Singh B, Wellstein A and Riegel AT. (2001). *Breast Cancer Res. Treat.*, **68,** 21–28.
Liu F, Hata A, Baker JC, Doody J, Carcamo J, Harland RM and Massague J. (1996). *Nature*, **381,** 620–623.
Massagué J and Chen YG. (2000). *Genes Dev.*, **14,** 627–644.
Massagué J and Weis-Garcia F. (1996). *Cancer Surv.*, **27,** 41–64.
McKenna NJ, Lanz RB and O'Malley BW. (1999). *Endocr. Rev.*, **20,** 321–344.
Na SY, Lee SK, Han SJ, Choi HS, Im SY and Lee JW. (1998). *J. Biol. Chem.*, **273,** 10831–10834.
Onate SA, Tsai SY, Tsai MJ and O'Malley BW. (1995). *Science*, **270,** 1354–1357.
Pouponnot C, Jayaraman L and Massague J. (1998). *J. Biol. Chem.*, **273,** 22865–22868.
Powell SM, Christiaens V, Voulgaraki D, Waxman J, Claessens F and Bevan CL. (2004). *Endocr. Relat. Cancer*, **11,** 117–130.
Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K, Kimura A, Nakamura Y, Inazawa J, Abe T and Yamagishi H. (2000). *Int. J. Cancer*, **89,** 217–223.
Shen X, Hu PP, Liberati NT, Datto MB, Frederick JP and Wang XF. (1998). *Mol. Biol. Cell.*, **9,** 3309–3319.
Shi Y, Hata A, Lo RS, Massague J and Pavletich NP. (1997). *Nature*, **388,** 87–93.
Shi Y and Massagué J. (2003). *Cell*, **113,** 685–700.
Snowden AW and Perkins ND. (1998). *Biochem. Pharmacol.*, **55,** 1947–1954.
Topper JN, DiChiara MR, Brown JD, Williams AJ, Falb D, Collins T and Gimbrone Jr MA. (1998). *Proc. Natl. Acad. Sci. USA*, **95,** 9506–9511.
Voegel JJ, Heine MJ, Zechel C, Chambon P and Gronemeyer H. (1996). *EMBO J.*, **15,** 3667–3675.
Wakefield LM and Roberts AB. (2002). *Curr. Opin. Genet. Dev.*, **12,** 22–29.
Workman JL and Kingston RE. (1998). *Annu. Rev. Biochem.*, **67,** 545–579.
Yanagisawa J, Yanagi Y, Masuhiro Y, Suzawa M, Watanabe M, Kashiwagi K, Toriyabe T, Kawabata M, Miyazono K and Kato SC. (1999). *Science*, **283,** 1317–1321.
Yuan W, Condorelli G, Caruso M, Felsani A and Giordano A. (1996). *J. Biol. Chem.*, **271,** 9009–9013.
Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B and Kern SE. (1998). *Mol. Cell*, **1,** 611–617.