Regulation of Transforming Growth Factor-β1–Induced Apoptosis and Epithelial-to-Mesenchymal Transition by Protein Kinase A and Signal Transducers and Activators of Transcription 3

Yanan Yang, Xinchao Pan, Weiwei Lei, Jun Wang, Jian Shi, Fuqiang Li, and Jianguo Song

Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

Abstract

Apoptosis and epithelial-to-mesenchymal transdifferentiation or transition (EMT) are crucial for normal development and body homeostasis. The alterations of these events are closely related to some pathologic processes, such as tumor formation and metastasis, fibrotic diseases of liver and kidney, and abnormal development of embryos. The mechanism that underlies the simultaneously occurring apoptosis and EMT induced by transforming growth factor-β (TGF-β) has not been well studied. In this report, we investigated the potential mechanism that underlies TGF-β1–induced apoptosis and EMT. TGF-β1–induced apoptosis and EMT were associated with the activation of protein kinase A (PKA) and signal transducers and activators of transcription 3 (STAT3). Inhibition of PKA by specific PKA inhibitor H89 or by PKA inhibitor peptide blocked STAT3 activation and suppressed TGF-β1–induced apoptosis and EMT. Furthermore, overexpression of a phosphorylation-deficient form of STAT3, but not wild-type STAT3, produced an inhibitory effect on TGF-β1–induced apoptosis and EMT. The results indicate that PKA is an upstream regulator for TGF-β1–induced STAT3 activation and plays an important role in TGF-β1–mediated apoptosis and EMT. These studies provided a new insight into the signaling mechanism underlying the apoptosis and EMT, which could be of importance in understanding some related physiologic and pathologic processes. (Cancer Res 2006; 66(17): 8617-24)

Introduction

Apoptosis and epithelial-to-mesenchymal transdifferentiation or transition (EMT) are two fundamental events essential to many physiologic and pathologic processes. Apoptosis is a cell suicide program characterized by a series of morphologic and biochemical changes (1), including the accumulation of sub-G_1 population, nuclear and DNA fragmentation, and, in most cases, caspase activation. Apoptosis could be triggered by various stimuli, including developmental, genetic, toxic, and survival and death factors. In the past decade, a great number of reports on apoptosis have emerged, leading to a tremendous advancement in the understanding of this phenomenon. In recent years, EMT has been received much attention. However, its regulatory mechanism has not been well understood. EMT is actively involved not only in tumor invasion, metastasis, and embryonic development but also in tissue homeostasis, wound healing, and fibrosis (2–6). The most apparent characteristic of EMT is the morphologic alterations from epithelial-to-mesenchymal phenotype, which is often accompanied by the dissolution of epithelial tight junctions, the loss of cell adhesion, down-regulated expression of some epithelial markers, as well as the acquisition of migratory and invasive properties. Along with these changes, the cytoskeleton of the cells was rearranged to render the cells spindle-like morphology.

Epithelial tight junctions form a functional and morphologic boundary on the cell surface and participate in the maintenance of epithelial integrity that protects multicellular organisms from the external environment (7, 8). Zonula occludens-1 (ZO-1) belongs to a family of membrane-associated guanylate kinase homologues that function as essential tight junctional plaque proteins. These plaque proteins can serve as direct links between the tight junction and the cytoskeleton (8, 9). During EMT, the loss of tight junctions and, accordingly, the delocalization of their structural components (e.g., ZO-1) from cell-cell contact was implicated in the depolarization of epithelial cells (10, 11).

Transforming growth factor-β (TGF-β) family members are pleiotropic factors implicated in diverse biological processes, such as embryonic development, tumorigenesis, inflammation, fibrosis, cell apoptosis, and proliferation (12–14). TGF-β was believed to contribute to the late-stage carcinoma by inducing EMT and promoting metastasis (4, 13, 15), although it functions as a potent growth inhibitor and apoptosis inducer for many types of epithelial cells.

TGF-β is able to induce multiple responses in hepatocytes, such as growth inhibition, apoptosis, and EMT (16, 17). Although TGF-β–induced EMT has been firstly reported a decade ago (18) and has become a focus of investigation in recent years, the mechanism of TGF-β–induced EMT remained not elucidated yet. Different family members of TGF-β may have completely different effect on EMT (19). It has been suggested that TGF-β–induced EMT in hepatocytes confers resistance to apoptosis (17). TGF-β1–induced concomitant apoptosis and EMT are two mutually exclusive processes. When some cells are undergoing apoptosis, some other cells are undergoing simultaneously EMT. Cells that undergo apoptosis will have no chance to undergo EMT. Likewise, when some cells undergo EMT, they escaped at least temporarily the TGF-β1–induced apoptotic response. Apoptosis and EMT have been shown to contribute differentially to the effects of TGF-β on tumor progression and embryonic development. Although it has been known that TGF-β1 can induce apoptosis and EMT in hepatocytes, little attention has been given to the signaling mechanism underlying these TGF-β–induced simultaneous responses, and it is not clear whether these different processes share some common cellular signaling events.

Note: Y. Yang and X. Pan contributed equally to this work.

Requests for reprints: Jianguo Song, Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China. Phone: 86-21-54921167; Fax: 86-21-54921167; E-mail: jgsong@sibs.ac.cn.

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Members of signal transducers and activators of transcription (STAT) family of transcriptional factors were originally identified as the critical mediators of cytokine responses and subsequently implicated in diverse external stimuli-initiated cellular signaling (20–22). STAT family members have been differentially implicated in the regulation of cell survival and apoptosis. It has been suggested that STAT1 functions in the suppression of cell growth and induction of cell apoptosis. STAT1 was shown to play an important role in the induction of cell apoptosis (23, 24). On the contrary, STAT5 has been considered to play a negative role in the regulation of cell apoptosis (25). It can be activated by growth factor receptors and facilitate the cell survival and growth by induction of several prosurvival proteins, such as Bcl family members. Controversial evidence about the role of STAT3 in cell survival and death has been reported. On the one hand, some growth factors and oncogenes can induce the activation of STAT3 and the subsequent expression of prosurvival proteins and cell cycle regulators, resulting in the cell proliferation and the inhibition of apoptosis (25–27). For this reason, STAT3 has been considered as an oncogene, and many types of tumor cells express activated forms of STAT3 (28). On the other hand, the proapoptotic function of STAT3 also has been recognized in both *in vitro* and *in vivo* models. For example, STAT3 was required for interleukin-6 (IL-6) or leukemia inhibitory factor–induced apoptosis of myeloid leukemia cells (29) and it was implicated in the apoptotic signaling during mammary gland involution (30, 31). It seemed that the role of STAT3 in the regulation of apoptosis could be dependent on the cell-specific cellular context and the type of stimuli. STAT3 activation by tyrosine phosphorylation, which is critical for its dimerization and nuclear translocation, represents its predominant activation mechanism (22). However, it was also reported recently that TGF-β–induced serine but not tyrosine phosphorylation of STAT3 was involved in mesoderm induction (32). The potential roles of STATs in other TGF-β responses remain completely unknown.

Protein kinase A (PKA) is a serine/threonine kinase widely implicated in the growth factor–mediated regulation of cell cycle and survival (33, 34). The holoenzyme of PKA consists of regulatory subunits and catalytic subunits. On activation, the catalytic subunits are released from the regulatory subunits and become competent to phosphorylate its cellular substrates at both the cytoplasm and the nucleus (35, 36). For example, a portion of activated PKA catalytic subunits translocate to the nucleus where they can directly phosphorylate and activate cyclic AMP–responsive element binding protein (CREB) transcriptional factor. Although it was shown that TGF-β is capable of activating PKA (37, 38), the role of PKA in TGF-β–mediated biological function, including apoptosis and EMT, still remains unidentified.

In this study, we investigated the potential mechanism of TGF-β1–induced apoptosis and EMT. TGF-β1 induces concomitant apoptosis and EMT, which are associated with increased PKA activity and the tyrosine phosphorylation of STAT3. Inhibition of PKA results in the suppression of TGF-β1–induced apoptosis and EMT. Inhibition of PKA also leads to the inhibition of TGF-β1–induced tyrosine phosphorylation of STAT3. In addition, transfection of dominant-negative STAT3 showed an inhibitory effect on TGF-β1–induced apoptosis and EMT. Our data suggest that PKA is an important regulator for STAT3 activation and is required for TGF-β1–induced apoptosis and EMT.

Materials and Methods

Materials. AML-12 murine hepatocytes were purchased from the American Type Culture Collection (Manassas, VA). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). H89 and BD-fmk were bought from Calbiochem (La Jolla, CA). Myristoylated protein kinase

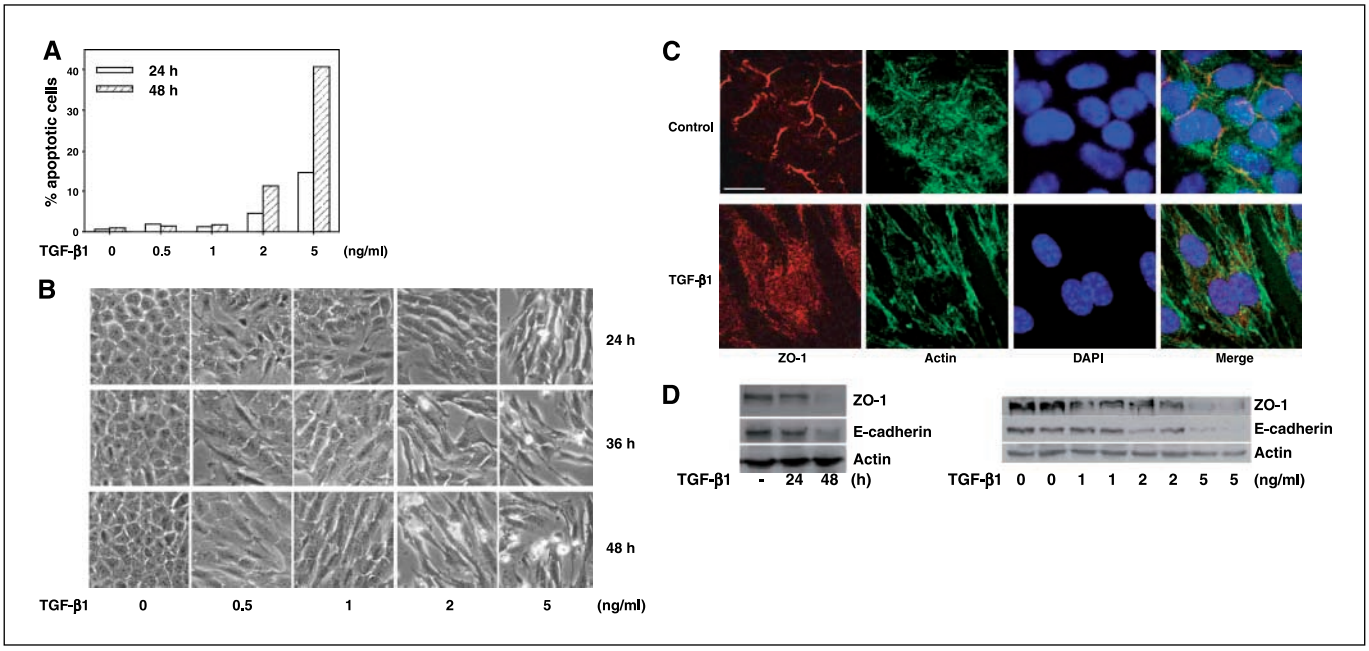

Figure 1. TGF-β1–induced apoptosis and EMT. Dose and time effects of TGF-β1 on apoptosis and EMT. *A,* apoptosis was determined by FACS assay in AML-12 cells. *Columns,* mean of three independent experiments; *bars,* SD. *B,* dose and time effects of TGF-β1 on EMT were examined by morphologic changes in AML-12 cells. *C,* EMT induced by TGF-β1 (2 ng/mL, 36 hours) was examined by immunostaining of ZO-1 (*red*) and β-actin (*green*). *Blue,* nuclei were stained by DAPI. *D,* time (5 ng/mL TGF-β1; *left*) and dose (48 hours; *right*) effects of TGF-β1 on the expression levels of ZO-1 and E-cadherin by immunoblotting.

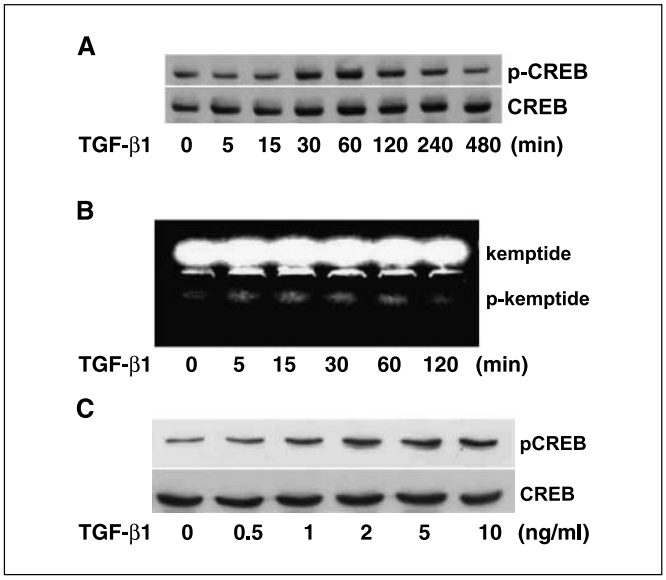

Figure 2. TGF-β1–induced PKA activation. PKA activation was determined by endogenous CREB phosphorylation (immunoblotting; *A*) or the *in vitro* phosphorylation of kemptide (*B*) in cells treated by TGF-β1 (5 ng/mL) in the indicated times. *C,* dose effect of TGF-β1 treatment (1 hour) on CREB phosphorylation (immunoblotting).

inhibitor (PKI) was purchased from Biomol (Plymouth Meeting, PA). Rabbit polyclonal antibodies against ZO-1 and PKA Cα, goat polyclonal antibodies for β-actin and PKI, horseradish peroxidase–conjugated anti-rabbit, mouse, and goat secondary antibody, and bovine anti-rabbit (Texas red conjugated), anti-mouse (FITC), and anti-goat (FITC) secondary antibodies, and avidin-biotin complex method (ABC) cytochemical staining kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against phosphorylated CREB, CREB, and STAT3 and mouse monoclonal antibody against phosphorylated STAT3 (Y705) were bought from Cell Signaling (Beverly, MA). Nitrocellulose membrane (HybondTM ECL) was bought from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). SuperSignal reagents were purchased from Pierce (Rockford, IL). Other reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Cell culture. AML-12 murine hepatocytes were cultured in a 1:1 mixture of DMEM and Ham's F12 containing 10% FCS (Life Technologies, Invitrogen) and supplied with insulin (5 μg/mL), transferrin (5 μg/mL), selenium (5 μg/mL), dexamethasone (40 ng/mL), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were incubated at 37°C in a humidified atmosphere of 10% CO_2. Experiments were done when cells reached 60% to 80% confluence.

Cell cycle analysis. Cell cycle was quantitatively determined by flow cytometry analysis as described previously (39). The percentage of cells with a sub-G_1 DNA content was taken as a measure of the apoptotic rate of the cell population.

Examination of morphologic change. The morphologic changes of the cells were observed under the inverted phase-contrast microscope (Olympus, Tokyo, Japan). The photographs were taken at 400× magnitude using Kodak (Xiamen, China) 200 films.

Immunofluorescence and immunocytochemical staining assays. Cells were grown on glass slides and treated as indicated. To terminate the reactions, the slides were quickly washed with PBS followed by fixing in 100% methanol at −20°C for 10 minutes. The samples were subjected to probing with appropriate primary and secondary antibodies (Texas red–conjugated anti-rabbit antibody, red fluorescence; FITC-conjugated anti-goat antibody, green fluorescence). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue fluorescence). The fluorescence was visualized under confocal microscopy (Leica, Mannheim, Germany). The immunocytochemical staining was done using an ABC kit under the manufacturer's instruction.

Preparation of cell lysates and immunoblotting. Cells were lysed in lysis buffer containing 50 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 50 mmol/L NaCl, 1% Triton X-100, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7{\cdot}10H_2O$, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 mmol/L Na_3VO_4, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Proteins (30 μg) were electrophoresed in SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were subsequently blocked with 5% skim milk and incubated with appropriate antibodies. Protein bands were visualized with SuperSignal reagents.

Biochemical subcellular fractionation. Cells were fractionated into subcellular components as follows: cells were lysed in lysis buffer [20 mmol/L HEPES (pH 7.0), 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.5% NP40, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF] and homogenized by 30 strokes in a glass homogenizer. The homogenate was centrifuged at 1,500 × *g* for 5 minutes to sediment the nuclei. The supernatant was then centrifuged at 15,000 × *g* for 10 minutes, and the resulting supernatant was the cytosolic fraction. The nuclear pellet was washed thrice and resuspended in lysis buffer containing 0.5 mol/L NaCl to extract nuclear proteins. The extracted material was precipitated at 15,000 × *g* for 20 minutes, and the resulting supernatant was the nuclear fraction.

Transfection. Cells in 35-mm plates were transfected with the plasmids as indicated using LipofectAMINE transfection reagent (Invitrogen) under the manufacturer's instruction. For transient transfection, the expression of the indicated plasmids was examined after 48-hour transfection. Stable transfection was done by G418 (800 μg/mL) selection following transient transfection.

Acridine orange/ethidium bromide staining of apoptotic morphology. Cells were stained by acridine orange (2 μg/mL) and ethidium bromide (2 μg/mL). Fluorescence was visualized immediately with a fluorescent microscope. The normal cells appear uniformly green, and apoptotic cells will be incorporated by ethidium bromide and therefore stained orange with condensed and often fragmented nuclei.

PKA kinase assay. Cells in 35-mm plates were lysed in 100 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 50 mmol/L NaCl, 1% Triton X-100, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7{\cdot}10H_2O$, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 mmol/L Na_3VO_4, and 1 mmol/L PMSF. Cell lysates were prepared and subjected to an *in vitro* PKA kinase assay using a kit from Promega under the manufacturer's instruction.

Cloning of mouse PKI. The cDNA of mouse PKI was amplified from a mouse testis cDNA library, and the primers used were designed as follows: 5′-GGCAAGCTTATGACTGATGTGGAAACTACG-3′ (sense) and 5′-GGCTCTAGATTAGCTTTCAGACTTGGCTGCTTC-3′ (antisense). The amplified PKI cDNA sequence was validated by sequencing and then cloned into a pcDNA3 vector.

DNA fragmentation assay. DNA fragmentation of apoptotic cells was done as described previously (40).

Statistical analysis. Results are presented as mean ± SD for the number of experiments indicated. Statistical analysis was made by Student's *t* test. Differences were considered significant at levels of <0.05 and <0.01.

Results

TGF-β1 induces concomitant cell apoptosis and EMT. TGF-β1 is a potent apoptosis inducer for many epithelial cell types, including hepatocytes. As shown in Fig. 1*A*, TGF-β1 induced strong apoptotic response in both time- and dose-dependent manners in AML-12 cells as examined by fluorescence-activated cell sorting (FACS) assay. Interestingly, besides apoptosis, some of the cells underwent a morphologic change characteristic of EMT, in which the cell shape changed from round into a spindle-like phenotype (Fig. 1*B*). The morphologic change was also dose and time dependent, which become pronounced in cells treated by 2 ng/mL TGF-β1 for 36 hours. To better characterize

TGF-β1–induced EMT, we examined the changes of tight junctions and cell skeleton by immunofluorescent staining of ZO-1 and β-actin, respectively. The results showed that localization of ZO-1 at cell-cell contact changed to a much wide-ranged intracellular distribution in response to TGF-β1 (Fig. 1*C*), indicating the loss or dissolution of tight junctions during EMT. TGF-β1–induced EMT was also associated with the cell skeletal rearrangement as revealed by β-actin staining (Fig. 1*C*). Further examination showed that TGF-β1 also significantly decreased the ZO-1 and E-cadherin expression levels in both time-dependent (Fig. 1*D, left*) and dose-dependent (Fig. 1*D, right*) manners. These results indicate that, although some cells are undergoing apoptosis, some other cells are undergoing EMT.

TGF-β1–induced apoptosis and EMT are associated with the PKA activation. Next, we explored the potential cellular signaling events involved in TGF-β1–induced apoptosis and EMT. We found that the phosphorylation of CREB, which serves as an endogenous substrate for activated PKA, was increased in response to TGF-β1 treatment (Fig. 2*A*). To confirm the activation of PKA, we did an *in vitro* PKA kinase assay using the specific substrate kemptide. The results showed that TGF-β1 also increased kemptide phosphorylation (Fig. 2*B*), suggesting a role of PKA in TGF-β1 signaling. Additional experiments showed the dose effect of TGF-β1 on PKA activation (Fig. 2*C*), in which a significant increase in CREB phosphorylation can be induced at the concentration of 1 to 2 ng/mL TGF-β1. Moreover, an increased nuclear translocation of PKA α-catalytic subunit (PKA Cα) in response to TGF-β1 treatment was observed by immunofluorescent staining assay (data not shown). These results showed the activation of PKA by TGF-β1.

PKA plays important role in TGF-β1–induced apoptosis and EMT. To test whether the activation of PKA contributed to TGF-β1–induced responses, we examined the effect of specific PKA inhibitor H89 on these events. As it was shown, H89

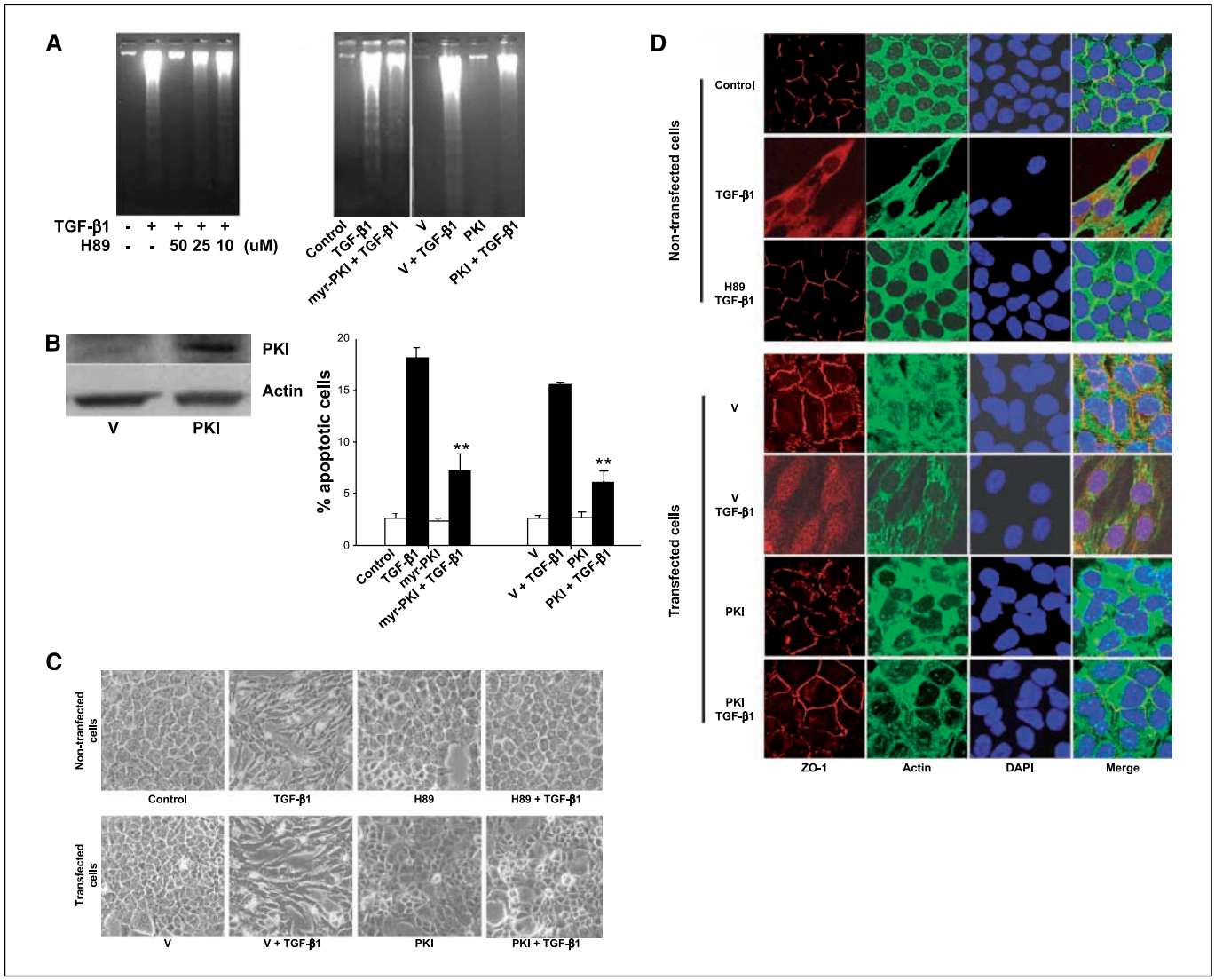

Figure 3. Role of PKA in apoptosis and EMT. *A,* cells were treated with TGF-β1 (5 ng/mL) for 24 hours, and apoptosis was determined by DNA fragmentation assay. *B, left,* expression of PKI in transfected cells by immunoblotting; *right,* apoptosis was determined by FACS assay. *Columns,* mean of three independent experiments; *bars,* SD. *C,* inhibition of PKA by H89 (50 μmol/L) or by PKI expression suppressed TGF-β1–induced (5 ng/mL, 48 hours) EMT as determined by morphologic changes. *D,* transfected cells were treated with TGF-β1 (2 ng/mL) for 36 hours. EMT was determined by immunostaining of ZO-1 and β-actin. Nuclei were stained by DAPI. *V,* cells transfected with pcDNA3 vector; *PKI,* cells transfected with pcDNA3 PKI; *myr-PKI,* myristoylated PKI.

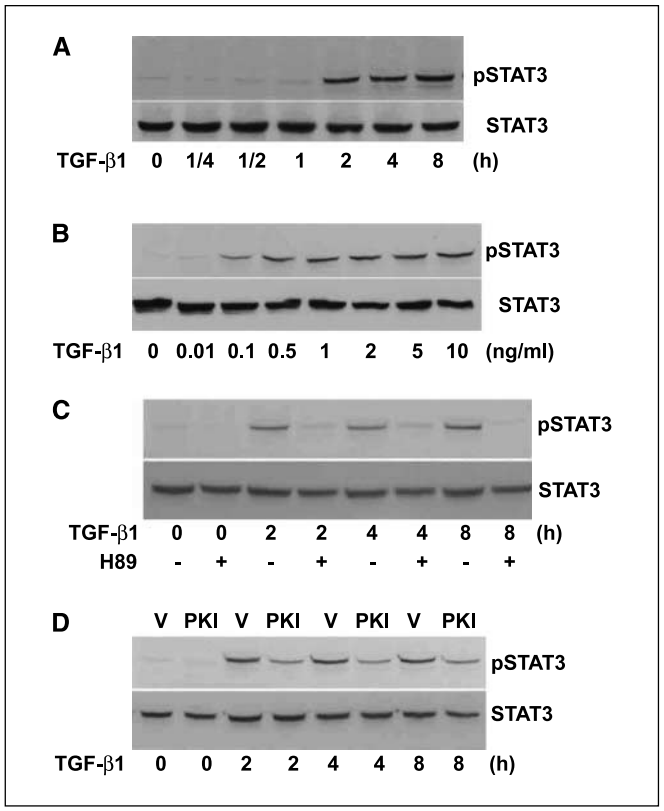

Figure 4. TGF-β1 induces the STAT3 tyrosine phosphorylation. Cells were treated with 5 ng/mL TGF-β1. *A,* STAT3 phosphorylation on Tyr^{705} residue (*pSTAT3*) was determined by immunoblotting. *B,* cells were treated with different concentrations of TGF-β1 for 4 hours, and STAT3 phosphorylation was determined by immunoblotting. *C,* STAT3 phosphorylation was determined by immunoblotting in cells treated with TGF-β1 (5 ng/mL) for the indicated times in the presence or absence of selective PKA inhibitor H89 (50 μmol/L). *D,* STAT3 phosphorylation in transfected cells (vector and PKI) treated with TGF-β1 (5 ng/mL) for the indicated times.

suppressed the TGF-β1–induced apoptosis in a dose-dependent manner as measured by DNA fragmentation assay (Fig. 3*A, left*). In line with this result, both the administration of exogenous cell-permeable myristoylated PKI and transfection of PKI (Fig. 3*B, top*), a PKA antagonist, also inhibited apoptosis as determined by DNA fragmentation (Fig. 3*A, right*) and FACS (Fig. 3*B, bottom*) assays. Similarly, inhibition of PKA also blocked TGF-β1–induced EMT as determined by examination of cell morphology (Fig. 3*C*), the ZO-1 localization, and β-actin distribution by immunofluorescent staining assay (Fig. 3*D*). These results showed that PKA is implicated in apoptosis and EMT induced by TGF-β1.

TGF-β1 induces STAT3 activation via PKA. Further investigations showed that TGF-β1 also induced the phosphorylation of STAT3 at Tyr^{705} residue, which was in both time-dependent (Fig. 4*A*) and dose-dependent (Fig. 4*B*) manners. Furthermore, we also detected the nuclear translocation of phosphorylated STAT3 through immunoblotting, immunofluorescent, and immunocytochemical assays (data not shown). Because both PKA and STAT3 were activated by TGF-β1 in AML-12 cells, we next examined the relationship of these two molecules. Interestingly, in the presence of selective PKA inhibitor H89, TGF-β1–induced phosphorylation of STAT3 was strongly suppressed (Fig. 4*C*), suggesting that STAT3 activation was mediated by PKA. To confirm this, we used PKI-transfected cells to examine the effect of inhibition of PKA on the STAT3 phosphorylation (Fig. 4*D*). The results showed that the expression of PKI also suppressed TGF-β1–induced STAT3 phosphorylation. Furthermore, the TGF-β1–induced nuclear translocation of STAT3 was also blocked by H89 or by the expression of PKI (data not shown). These data indicate that TGF-β1–induced activation of PKA plays a critical role in the activation of STAT3.

STAT3 is involved in TGF-β1–induced apoptosis and EMT. To investigate the potential role of STAT3 in TGF-β1–mediated apoptosis and EMT, we transfected the cells with FLAG-tagged wild-type (wtSTAT3) and phosphorylation-deficient (dnSTAT3) forms of STAT3 (Fig. 5*A, top*). The results showed that dnSTAT3 suppressed TGF-β1–induced apoptosis as determined by FACS assay (Fig. 5*A, bottom*), DNA fragmentation assay (Fig. 5*B*), or acridine orange/ethidium bromide staining of apoptotic morphology (data not shown). In addition, TGF-β1 failed to induce EMT in cells overexpressing dnSTAT3 as determined by examination of cell morphology (Fig. 5*C*) or the intracellular localization of ZO-1 and β-actin (data not shown). As shown in Fig. 5*D*, transfection of dnSTAT3 also reversed TGF-β1–mediated down-regulation of ZO-1 and E-cadherin expressions. The results indicated that TGF-β1–induced activation of STAT3 by tyrosine phosphorylation plays a role in the induction of apoptosis and EMT.

TGF-β1–induced EMT is an independent event. Because TGF-β1–induced apoptosis is dependent on caspase activation (40), we examined the effect of caspase inhibition on TGF-β1–induced EMT and apoptosis to confirm that TGF-β1–induced EMT is an independent event from apoptosis. The results showed that a broad-spectrum caspase inhibitor BD-fmk inhibited apoptosis (Fig. 6*A*) but had no effect on the TGF-β1–induced morphologic change (Fig. 6*B*). BD-fmk also had no effect on the changes of ZO-1 and E-cadherin expression levels induced by TGF-β1 (Fig. 6*C*). These results suggest that the caspase activation is not related with EMT and TGF-β1–induced EMT is an independent concomitant event.

Discussion

As a multifunctional factor, TGF-β is involved in the regulation of many biological processes, including the apoptosis and EMT. Much less attention has been given to the ability of TGF-β to induce different responses in the same cell types, and the mechanism underlying these responses has not been well understood. Because TGF-β1 can induce concomitantly apoptosis and EMT not only in AML-12 hepatocytes but also in Mv1Lu (mink lung epithelial cells) and Madin-Darby canine kidney cells (data not shown), we therefore studied the potential mechanism through which TGF-β1 induced apoptosis and EMT and focused in this study on the role of some signaling molecules in TGF- β1–induced responses in AML-12 hepatocytes.

TGF-β1–induced EMT was observed first by morphologic changes of the cells. It was subsequently determined by examining the changes of cellular localization of ZO-1 and β-actin or the expression levels of E-cadherin and ZO-1. The disruption of tight junctions, which is an indicator of loss or attenuation of epithelial polarity, is a hallmark of EMT (41). Our results showed that TGF-β1–induced morphologic changes of cells are associated with the delocalization of tight junction protein ZO-1 from cell-cell contact. Consistent with the above evidence, we observed that TGF-β1–induced EMT is associated with a corresponding increase in the ability of cell migration (data not shown), supporting the notion that the plasticity of epithelial cells to differentiate into

fibroblastic-like cells is closely related with their migration capacity (42, 43). This plasticity of epithelial cells is important for organogenesis in embryonic development and is implicated in certain tumor invasiveness.

In recent years, some signaling molecules other than Smads are found to be involved in TGF-β signaling. For example, p38 mitogen-activated protein kinase was previously been shown to play an important role in TGF-β1–induced apoptosis in AML-12 cells (44). It is likely that there can be interactions between Smads and other signaling molecules through a feedback or other mechanism because TGF-β–mediated effects are generally the results of a long-term process that generally takes at least several hours. The interaction between Smads and other signaling molecules has been supported by emerging evidence. For example, activated Smads were found to be able to form a complex with PKA, which is critical for TGF-β1–mediated PKA activation (38). In this study, we also observed a direct interaction between PKA and Smads (data not shown), implying a possibility of the involvement of the similar mechanism in TGF-β1–induced apoptosis and EMT. More importantly, our data showed that the activation of PKA is important for the induction of apoptosis and EMT by TGF-β1. These observations provided a new insight into the signaling mechanism of TGF-β1–induced apoptosis and EMT. Meanwhile, it also presents a novel function of PKA that has not been identified before. As it has been reported that PKA signal facilitates *Snail2* induction and EMT partly by promoting Sox9 and Snail2 function in response to bone morphogenetic protein stimulation in avian neural crest (45), TGF-β1–induced EMT involving PKA activation is therefore supposed to play an important role in developmental process. PKA may represent a common signaling event in TGF-β1–induced multifunctional processes. Thus, it is important to know how TGF-β1 activated PKA, whether the complex formation between Smad and PKA suggests a mechanism through which TGF-β1 regulates the PKA activity.

Among STAT transcriptional factors, STAT3 has been shown to play a role in various biological processes, including proliferation, differentiation, and apoptosis (20, 22). The activity of STAT3 can be regulated by phosphorylation at tyrosine and/or serine residues within their COOH termini. Generally, the tyrosine phosphorylation is required for the dimerization and nuclear translocation of these factors, whereas the serine phosphorylation is involved in the enhancement of their transcriptional activity and interaction with other signaling molecules. The relationship between TGF-β1 signaling and STAT3 has not been well understood. IFN-γ-mediated STAT3 activation was shown to be implicated in the inhibition of TGF-β1–induced expression of fibronectin and plasminogen activator inhibitor-1 (46). On the other hand, TGF-β1 can inhibit IL-6-induced tyrosine phosphorylation of STAT1 and STAT3 and suppression of intercellular adhesion molecule-1 expression (47). In the present study, activation of STAT3 as determined by an increase in STAT3 tyrosine phosphorylation was observed. Subsequently, STAT3 was shown to be important for TGF-β1–induced concomitant apoptosis and EMT. No significant change in serine phosphorylation was observed at the same time (data not shown). The data indicate that TGF-β1 regulates STAT3 through tyrosine phosphorylation in AML-12 cells. Consistent with our observation, the implication of STAT in the induction of

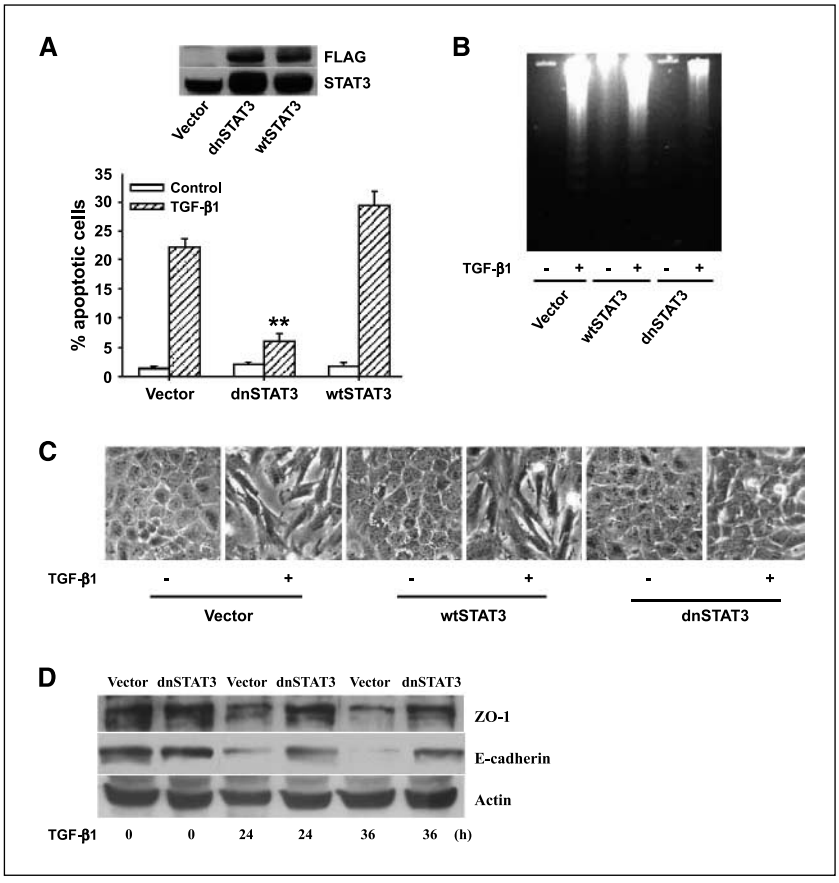

Figure 5. Role of STAT3 in apoptosis and EMT. AML-12 cells were transfected with FLAG-tagged phosphorylation-deficient (*dnSTAT3*) and wild-type (*wtSTAT3*) forms of STAT3. *A, top,* expression levels of dnSTAT3 and wtSTAT3; *bottom,* transfected cells were treated with TGF-β1 (5 ng/mL) for 24 hours, and apoptosis was analyzed by FACS assay. *Columns,* mean of three independent experiments; *bars,* SD. *B,* transfected cells were treated with TGF-β1 (5 ng/mL) for 24 hours, and apoptosis was analyzed by DNA fragmentation assay. *C,* EMT was examined by morphologic changes. *D,* expression levels of ZO-1 and E-cadherin were determined by immunoblotting in cells transfected with vector or dnSTAT3. Transfected cells were treated with TGF-β1 (2 ng/mL) for 36 hours.

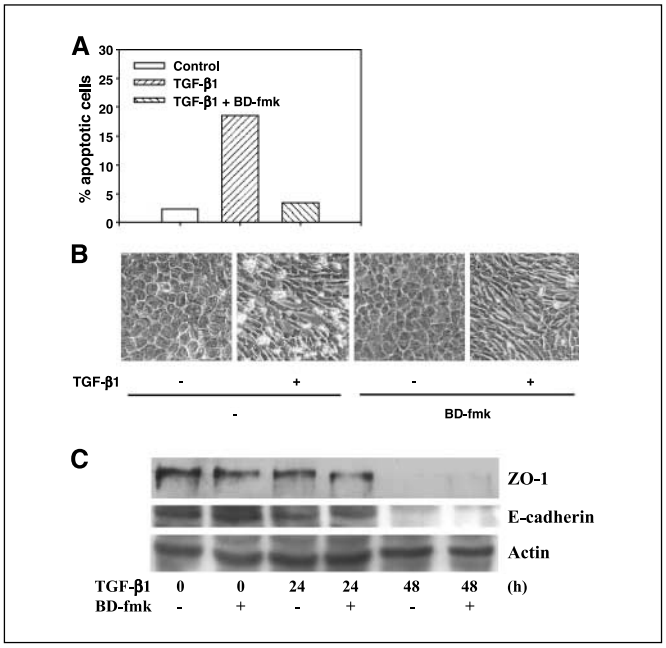

Figure 6. TGF-β1–induced apoptosis and EMT are independent events. Effect of caspase inhibitor BD-fmk on TGF-β1–induced apoptosis and EMT. *A,* apoptosis was shown by FACS assay. Cells were treated with TGF-β1 (5 ng/mL) for 24 hours in the presence or absence of BD-fmk (100 μmol/L). *B,* effect of BD-fmk on TGF-β1–induced EMT was determined by morphologic changes. Cells were treated with TGF-β1 (5 ng/mL) for 48 hours in the presence or absence of BD-fmk (100 μmol/L). *C,* ZO-1 and E-cadherin expression levels by immunoblotting were shown in cells treated with TGF-β1 (5 ng/mL) for 24 and 48 hours in the presence or absence of BD-fmk.

apoptosis and EMT in other cases has been reported in the past several years (23, 30, 48).

It is not clear how STAT3 was activated by TGF-β1 and whether Smad molecules are directly involved in the activation of it. The result that TGF-β1–induced STAT3 activation was suppressed by inhibition of PKA indicates that PKA plays a role in the control of TGF-β1 signaling, which is associated with the modulation of STAT3 activity. PKA is a serine/threonine protein kinase that cannot directly catalyze the tyrosine phosphorylation, so there must be other signaling molecules that can serve as direct upstream regulator of STAT3. However, it is presently not clear which molecule downstream of PKA is directly responsible for the phosphorylation of STAT3. Src tyrosine kinase has been implicated in the tyrosine phosphorylation of STAT3 (22, 49). Interestingly, we also detected the interaction of c-Src and STAT3, and the TGF-β1–induced tyrosine phosphorylation of STAT3 was completely suppressed in the presence of Src inhibitor protein phosphatase 1 (data not shown). These observations suggest that c-Src is likely one of such candidate molecules mentioned above. The data also provided new evidence supporting a notion that a complex signaling network is involved in accomplishing multiple functions of TGF-β1. STAT3 can be activated by epidermal growth factor (EGF) in several types of cells, and it is also reported that EGF can inhibit TGF-β1–induced apoptosis but facilitate EMT (50). It is possible that some molecules that involved in EGF signaling may play a role in TGF-β1–induced apoptosis and EMT. We also found that TGF-β1–induced apoptosis and EMT are associated with Smad2 phosphorylation (data not shown), which suggested that Smad pathway may be involved in these two events. Whether PKA and STAT3 activation are dependent on Smad pathway and if PKA and STAT3 have the same downstream effectors of EMT and apoptosis remains to be characterized.

The present data showed that TGF-β1–induced simultaneously occurring apoptosis and EMT are distinguishable events that may be regulated via different mechanisms. Interestingly, our results showed that these two distinct phenomena are under the control of PKA. Therefore, there could be different downstream pathways used by PKA and STAT3 to determine the differential responses of the cells to TGF-β1. The results also suggested that PKA and STAT3 could function as critical points of the cross talk between different TGF-β1 responses.

The role of TGF-β in cancer formation and progression has been a major focus of interest and studies. TGF-β was considered to have two "faces" in its relationship with tumor formation and progression and was therefore been described as a "double-edged sword." The fact that TGF-β1 induces apoptosis and EMT suggests that the different "faces" of TGF-β can be seen under the same condition. The data also provided an explanation on the significance for the dual effect of TGF-β (apoptosis and EMT) in the development of multicellular organism because both apoptosis and EMT are essential for normal development and their dysregulation has been implicated in various pathologic processes. It is possible that TGF-β in reality shows both or different faces in all stages of cancer development as well as in embryogenesis, and its sum total or unified effects lie on cellular context and specific state and cellular environment, which confers the flexibility of cellular responses. TGF-β1-induced concomitant apoptosis and EMT represent a case in which different fates were induced for the same cell type in response to an identical stimulus. Because apoptosis and EMT are critical for development, body homeostasis, and tumor metastasis, it is evident that, in future work, the further identification of the underlying mechanism for these TGF-β1–induced events could be helpful for understanding the seemingly mystic roles of TGF-β.

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References

1. Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 2000;45:528–37.
2. Boyer B, Vallés AM, Edme N. Induction and regulation of epithelial-mesenchymal transitions. Biochem Pharmacol 2000;60:1091–9.
3. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003;112:1776–84.
4. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442–54.
5. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol 2003;15:740–6.
6. Prindull G, Zipori D. Environmental guidance of normal and tumor cell plasticity: epithelial mesenchymal transitions as a paradigm. Blood 2004;103:2892–9.
7. Morita K, Miyachi Y. Tight junctions in the skin. J Dermatol Sci 2003;31:81–9.
8. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 2004;286:C1213–28.

9. Matter K, Balda MS. Signalling to and from tight junctions. Nat Rev Mol Cell Biol 2003;4:225–36.

10. Grünert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol 2003;4: 657–65.

11. Shook D, Keller R. Mechanisms, mechanics, and function of epithelial-mesenchymal transitions in early development. Mech Dev 2003;120:1351–83.

12. Massagué J. How cells read TGF-β signals. Nat Rev Mol Cell Biol 2000;1:169–78.

13. Derynck R, Akhurst RJ, Balmain A. TGF-β signaling in tumor suppression and cancer progression. Nat Genet 2001;29:117–29.

14. Akhurst RJ. TGF-β signaling in health and disease. Nat Genet 2004;36:790–2.

15. Roberts AB, Wakefield LM. The two faces of transforming growth factor-β in carcinogenesis. Proc Natl Acad Sci U S A 2003;100:8621–3.

16. Spagnoli FM, Cicchini C, Tripodi M, Weiss MC. Inhibition of MMH (Met murine hepatocyte) cell differentiation by TGFβ is abrogated by pre-treatment with the heritable differentiation effector FGF1. J Cell Sci 2000;113:3639–47.

17. Valdés F, Alvarez AM, Locascio A, et al. The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor-β in fetal rat hepatocytes. Mol Cancer Res 2002;1:68–78.

18. Miettinen PJ, Ebner R, Lopez AR, Derynck R. TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J Cell Biol 1994;127:2021–36.

19. Zeisberg M, Hanai J, Sugimoto H, et al. BMP-7 counteracts TGFβ1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 2003;9:964–8.

20. Williams JG. STAT signalling in cell proliferation and in development. Curr Opin Genet Dev 2000;10: 503–7.

21. Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances, and future challenges. Gene 2002;285:1–24.

22. Levy DE, Darnell JE, Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3: 651–62.

23. Chin YE, Kitagawa M, Kuida K, Flavell RA, Fu XY. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Mol Cell Biol 1997;17:5328–37.

24. Kumar A, Commane M, Flickinger TW, Horvath CM, Stark GR. Defective TNF-α-induced apoptosis in Stat1 null cells due to low constitutive levels of caspases. Science 1997;278:1630–2.

25. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 2002;109:S121–31.

26. Calò V, Migliavacca M, Bazan V, et al. STAT proteins: from normal control of cellular events to tumorigenesis. J Cell Physiol 2003;197:157–68.

27. Bhattacharya S, Ray RM, Johnson LR. Integrin β_3 mediated Src activation regulates apoptosis in IEC-6 cells via Akt and STAT3. Biochem J 2006;397:437–47.

28. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474–88.

29. Minami M, Inoue M, Wei S, et al. STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line. Proc Natl Acad Sci U S A 1996;93:3963–6.

30. Abell K, Bilancio A, Clarkson RW, et al. Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition. Nat Cell Biol 2005;7:392–8.

31. Chapman RS, Lourenco PC, Tonner E, et al. Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev 1999;13:2604–16.

32. Ohkawara B, Shirakabe K, Hyodo-Miura J, et al. Role of the TAK1-NLK-STAT3 pathway in TGF-β–mediated mesoderm induction. Genes Dev 2004;18:381–6.

33. Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Prolif 2003;36:165–75.

34. Stork PJS, Schmitt JM. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 2002;12:258–66.

35. Barradeau S, Imaizumi-Scherrer T, Weiss MC, Faust DM. Intracellular targeting of the type-I regulatory subunit of cAMP-dependent protein kinase. Trends Cardiovasc Med 2002;12:235–41.

36. Taylor SS, Yang J, Wu J, Haste NM, Radzio-Andzelm E, Anand G. PKA: a portrait of protein kinase dynamics. Biochim Biophys Acta 2004;1697:259–69.

37. Wang L, Zhu Y, Sharma K. Transforming growth factor-β1 stimulates protein kinase A in mesangial cells. J Biol Chem 1998;273:8522–7.

38. Zhang L, Duan CJ, Binkley C, et al. A transforming growth factor β-induced Smad3/Smad4 complex directly activates protein kinase A. Mol Cell Biol 2004;24: 2169–80.

39. Zhao S, Yang Y, Song JG. Ceramide induces caspase-dependent and -independent apoptosis in A-431 cells. J Cell Physiol 2004;199:47–56.

40. Yang Y, Zhao S, Song JG. Caspase-dependent apoptosis and -independent poly-(ADP-ribose) polymerase cleavage induced by transforming growth factor-β1. Int J Biochem Cell Biol 2004;36:223–34.

41. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 2005;17: 548–58.

42. Friedl P. Prespecification and plasticity: shifting mechanisms of cell migration. Curr Opin Cell Biol 2004;16: 14–23.

43. Janda E, Lehmann K, Killisch I, et al. Ras and TGFβ cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. J Cell Biol 2002;156:299–313.

44. Liao JH, Chen JS, Chai MQ, Zhao S, Song JG. The involvement of p38 MAPK in transforming growth factor-β1-induced apoptosis in murine hepatocytes. Cell Res 2001;11:89–94.

45. Sakai D, Suzuki T, Osumi N, Wakamatsu Y. Cooperative action of Sox9, Snail2, and PKA signaling in early neural crest development. Development 2006; 133:1323–33.

46. Giannopoulou M, Iszkula SC, Dai C, et al. Distinctive role of Stat3 and Erk-1/2 activation in mediating interferon-γ inhibition of TGF-β1 action. Am J Physiol Renal Physiol 2006;290:F1234–40.

47. Walia B, Wang L, Merlin D, Sitaraman SV. TGF-β down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. FASEB J 2003;17: 2130–2.

48. Yamashita S, Miyagi C, Fukada T, et al. Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. Nature 2004;429: 298–302.

49. Frame MC. Newest findings on the oldest oncogene; how activated Src does it. J Cell Sci 2004;117: 989–98.

50. Docherty NG, O'Sullivan OE, Healy DA, et al. TGF-β1–induced EMT can occur independently of its proapoptotic effects and is aided by EGF receptor activation. Am J Physiol Renal Physiol 2006;290: F1202–12.