Study on the mechanism of cancer cell death induced by TGFβ1, TGFβ2 downregulation

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Study on the mechanism of cancer cell death induced by TGFβ1, TGFβ2 downregulation

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ABSTRACT

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TGF-β signaling has been increasingly recognized as a key driver in cancer. Unlike its tumor suppressor function in normal tissue, TGF-β activation incites tumor progression in cancer tissue and an increase in TGF-β expression often correlates with the malignancy of many cancers.

In this study, we tried to unravel the mechanism of TGF-β downregulation-induced by using adenovirus expressing short hairpin RNA against transforming growth factor-β1 or β2 (TGF-β1/2). Notably, we found that TGF-β downregulation could increase the phospho-p38 and phospho-JNK expression, also decrease the survival molecule such as phospho-Akt, phospho-Src, phospho-Stat3 and phospho-p65. Consistent with the increase of phosphor-p38 and p-JNK, the ASK1 phosphorylation (which means ASK1 activation) and reactive oxygen species(ROS) production were also increased in response to TGF-β downregulation, whereas gene expression of Trx and GSTM1 known to be inhibitory binding proteins to ASK1 were decreased. In

addition, interactions between GSTM1 and ASK1 or Trx and ASK1 were also decreased. This decrease in Trx and GSTM1 expression was likely to be related to the translocation of Smad complex proteins as a main mediator of canonical signalling pathway of TGF-β playing a tumor-promoting role by transcriptional activation of target genes such as Trx or GSTM1. However, ROS was not directly related to the transcriptional repression of Trx or GSTM1, while inducing dissociation of Trx and GSTM1 from ASK1 activation followed by tumor cell death. Morevoer, ASK1 inhibition with siASK1 or overexpression of a dominant-negative kinase-inactive mutant of ASK1(ASK1-KM) rescued cell death. In addition, p38 MAPK/JNK activation was also inhibited by siASK1 or ASK1-KM, suggesting that ASK1 signaling via p38 MAPK/JNK activation was the main pathway of adenovirus-expressing shTGF- β1 or 2 induced cell death.

Taken together, our findings demonstrate that treatment with adenovirus expressing shRNA of TGF-β1 or 2 can cause cell death via ASK1 activation, which was associated with the reduction of Trx and GSTM1 gene expression and dissociation of the Trx/GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes.

Key words: TGF-β1, TGF-β2, ROS, ASK1, adenovirus, cell death

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I. INTRODUCTION

Cancer is one of the most common diseases worldwide. While North American populations are twice as likely to develop cancer than those in Asia, the death rate in Asia was twice as high as that in North America.[1] Several modalities currently exist to treat cancer, including surgery, chemotherapy and radiation therap. Surgical resection is often used to remove a cancer in its entirety, however, many tumors have the tendency to spread to adjacent areas. In the cases, patients typically undergo chemotherapy and radiotherapy, which have known side-effects.[2] As such, gene therapy could serve as a means to treat cancer without the adverse effect on patients.[3]

Gene therapy is the delivery of nucleic acid polymers into a patient's

cells to treat disease.[4] The most common form of gene therapy uses DNA that encodes a functional, therapeutic gene to replace its endogenous mutated counterpart. The polymer molecule is packaged with viral or non-viral “vectors” for cellular uptake. For example, recombinant adenovirus can be grown to high titers and has a relatively high capacity for transgene insertion, usually without incorporation of viral DNA into the host cell genome.[5, 6] Moreover, the use of oncolytic adenovirus in gene therapy will not damage normal cells, but can be engineered to induce tumor-specific cell lysis.[5, 6, 7] During viral infection adenovirus virion particle to the cell surface occurs through binding of the fiber knob to the coxsackievirus B and adenovirus receptor (CAR).[6] Therefore, effective therapeutic gene delivery can be induced by using the adenoviral vector construct without any further engineering.[8, 9, 10] In addition, this vector can be used to transport various types of genes into the cell, without discrimination.[11, 12, 13]

TGF-β, a secreted cytokine, plays a multi-faceted role in tumorigenesis.[14] Interestingly, it functions as a tumor suppressor by restraining cell proliferation and immortalization, while encouraging apoptosis. Alternatively, TGFβ can also act as a promoter of tumor metastasis, such as induction of epithelial-mesechymal transitinon (EMT),

cell adhesion, migration, invasion, chemoattraction, and tumor metastasis.[15, 16, 17, 18, 19, 20] Notably, some human tumors become resistant to the effects of TGF-β as a result of genetic and epigenetic changes, whereas others are subject to pro-oncogenic pathway activation such as MAPK, PI3K, Ras, and c-MYC that can override any growth inhibitory signaling pathways.[21, 22]

The TGF-β receptor is a heteromeric cell-surface complex comprised of specific Type I and II transmembrane serine/ threonine kinases, which is highly expressed by tumor cells.[23] Ligand-induced receptor activation induces a temporary interaction between the TβRI receptor and Smad2/3 subsequently stabilized by the FYE protein SARA. TβRI phosphorylates the C-terminals of Smad2/3, resulting in their dissociation from the receptor and Smad4 recruitment. The Smad2/3/4 complex then translocates into the nucleus and interacts with the promoter with the transcription factors with sequence-specific DNA binding to regulate gene expression. Smad-mediated gene expression is controlled by several intracellular signaling pathways, including the c-Jun N-terminal kinase(JNK)/p38 MAP kinase and β-catenin/Wnt signaling. [24, 25]

TGFβ consists of three isoforms (TGFβ1/2/)3, each encoded by a

different genes.[26] Specifically, TGF-β1 regulates various immune responses depending on the cell type and development stage. In most cases, TGF-β1 is secreted by immune cells (or leukocytes).[27] Some T cells actions are inhibited by TGF-β1 released from the other T cells.[28] Likewise, TGF-β1 hinder the secretion of cytokines, such as interferon-γ(IFN-γ), tumor necrosis factor-alpha (TNF-α), and various interleukins(ILs). Alternatively, TGF-β1 acts to attenuate B cell proliferation while promoting apoptosis.[29] TGF-β2 distinguishes itself through its suppressive effects on early interleukin-dependent T cell tumors. In the advanced cancer stage, TGF-β levels were significantly higher, especially as TGF-β2. As is revealed by earlier research, poorer prognosis accounts for the increased expression level of TGF-β1 and TGF-β2 proteins.[30, 31] In a more general context, TGF-β also regulates cell proliferation, differentiation, angiogenesis, and wound healing and regulatory T cell activity.[32, 33] Moreover, for some immune response of certain cell types, such as NK cells, dendritic cells, macrophage and T cells are inhibited by cancer cells with the help of TGF-β signaling.[34] Collectively, these lines of evidence support TGF-β as a cancer target and enhance anti-tumor immunity.

In previous studies, we designed an adenovirus delivered TGF-β1

shRNA and TGF-β2 shRNA, to reduce TGF-β1/2expression, respectively. Antitumor effects were tested by adenovirus delivered TGF-β1 shRNA and TGF-β2 shRNA in various tumor cells, among the tumor cells with increased cell death the phospho-p38 and phospho-JNK expression were increased, also ROS production was increased. In addition, TGF-β signals via the conserved MAPK pathway including extracellular-related kinase 1/2 (ERK1/2 or p44/42 MAPK), c-Jun N-terminal kinase (JNK) and p38 MAPK to regulate cell proliferation, differentiation, survival and apoptosis[35] MAPKs also respond to various forms of extracellular stress, such as cytokines ultraviolet irradiation, heat shock, and osmotic stress.[36]

In response to various extracellular stimuli, such as TGF-β, intracellular signal transduction is activated. p38 MAPK can control gene expression to alter the cell growth, and apoptosis. For this reason, p38 MAPK has been considered a leading molecular target in cancer therapy.[37, 38]

Apoptosis signal-regulating kinase 1(ASK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates JNK and p38 by direct phosphorylation primarily in response to cytotoxic stressors such as tumor necrosis factor (TNF), Fas ligand, and reactive oxygen species (ROS) to accelerate apoptosis. [39, 40, 41, 42] It has been documented that the

mechanisms of MAPK stimulates apoptosis via Bcl-2 family proteins, and caspase family proteins in cancer cells.[43]

ROS including the superoxide anion radical ($O_2^{-\cdot}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($^{\cdot}OH$) are by products of oxygen metabolism.[44] ROS play a role in the central cellular process that is part of the development of a cancer cell, proliferation, apoptosis and senescence.[45]

Thioredoxin (Trx) is expressed by all living organisms. A Trx-ASK1 complex is generated with the integration of Trx and ASK1. Such complex renders the expression of ASK1 protein inactive.[46] ROS induces the dissociation between Trx and ASK1, which leads to the activation of the ASK1/JNK signaling pathway and subsequent increase of apoptosis.[47, 48] Alternatively, Glutathione S-transferase Mu 1(GSTM1) interacts with the ASK1 N-terminals to enhance oxidative stress-induced ASK1-dependent apoptosis.[49] GSTM1/ASK1 complex is dissociated under oxidative stress, then cause the activation of ASK1.[50]

In this research, we designed adenoviruses delivering TGF-β1 shRNA or TGF-β2 shRNA to reduce TGF-β1 or 2 expression and found that they can cause tumor cell death by inducing ASK1 activation and consequent p38 and JNK activation. The ASK1 activation was found to

be deeply related to both the reduction of Trx and GSTM1 gene expression and dissociation of Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes.

II. MATERIALS AND METHODS

1. Cell culture

A375, HPAC were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) and maintained in a 37 ℃ humidified atmosphere containing 5% CO_2. The medium was changed every 2–3 days after transfection.

2. Plasmids and recombinant proteins

HA-tagged ASK1 wild type was constructed in pCDNA3.1 plasmid (Invitrogen, Carlsbad, CA). A kinase-inactive form of ASK1 (K709M) was introduced into wild type of ASK1 with the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, SF, USA) using pcDNA3-HA-ASK1 as a template. The sense and antisense primer s were used 5′-gcaaccaagtcagaattgctattagggaaatcccagagagagac-3′ and 5′-gtctctctctgggatttccc taatagcaattctgacttggttgc-3′ for K709M. The small interfering RNA (siRNA) against ASK1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

3. Construction of adenoviral vectors

Human TGFβ1 and TGFβ2 shRNAs were generated with annealing

oligonucleotides subcloned into BamHI/HindⅢ-digested pSP72ΔE3-U6 shuttle vector termed pSP72ΔE3-U6-shTGFβ1, pSP72ΔE3-U6-shTGFβ2, respectively. The vectors were linearized by XmnI digestion, and co-transformed into *Escherichia.coli* BJ5183 with SpeI-digested adenoviral vector (dl324-IX) for homologous recombination. Viruses were defined as follows.

4. Name of recombinant adenovirus

Ad-NC: Ad-IX-ΔE1B, control virus

Ad-shTGFβ1: Ad-IX-ΔE1B-ΔE3-U6-shTGFβ1, virus expressing shRNA of human TGFβ1

Ad-shTGFβ2: Ad-IX-ΔE1B-ΔE3-U6-shTGFβ2, virus expressing shRNA of human TGFβ2

5. MTS viability assay

Cell viability was assessed with a CellTiter 96® Aqueous Assay kit (Promega, Madison, WI, USA) that contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS) is bioreduced by metabolically active cells in the presence of an electron coupling reagent (phenazineethosulfate; PES). Assays were performed 48 hr after adenovirus infection with A375 or HPAC cells seeded in 96-well plates. Absorbance at 490 nm was used to measure cell viability.

6. Western blot analysis

Cells were lysed in 1X Laemmli lysis buffer (62.5mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 0.002% bromophenol blue) and protein concentrations determined with BCA Protein Assay Kit (Thermo Scientific, Fremont, CA, USA). The protein samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electro transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were detected with anti-phoshoAkt (pAkt), anti-phosphoSrc (pSrc), anti-phosphoSTAT3(pSTAT3), anti-phosphoP65 (pP65), anti-Smad2(Smad2), anti-phosphoSmad2 (pSmad2), anti-ASK1, anti-phosphoASK1 (pASK1), anti-Smad3 (Smad3), anti-phosphoSmad3 (pSmad3), anti-phosphoERK (pERK), anti-phoshoP38 (pP38), anti-phosphoJNK (pJNK), anti-P65, anti-ERK, anti-Akt, anti-STAT3, anti-JNK, anti-Src and anti-P38, which were purchased from Cell Signaling Technology (Danvers, MA, USA), anti-phosphoHSP27(pHSP27), anti-HSP27(HSP27), anti-Trx(Trx), anti-GSTM1(GSTM1), anti-AP1(AP1), anti-Sp1(Sp1), anti-Smad4(Smad4), anti-GAPDH came from Santa Cruz Biotechnology (Dallas, TX, USA). Immunoreactive bands were visualized by chemi-luminescence or fluorescent imaging(Syngene, Cambridge, UK).

7. Real-time polymerase chain reaction(RT-PCR)

Total RNA was isolated from cells with standard Trizol (Life Technologies, Carlsbad, CA, USA)/chloroform extraction. RNA concentration was determined

with a Nanodrop 2000 (Thermo Scientific). RT-PCR was performed with the Power SYBR Green RNA-to-CT 1-Step Kit (Life Technologies) in reaction mixtures containing the reverse transcriptase enzyme mix, reverse transcription PCR mix, forward primer, reverse primer, RNA template and nuclease-free water. Human TGFβ1 cDNA was amplified using the forward primer, 5'-TTGCTTCAGCTCCACAGAGA -3', and the reverse primer: 5'-TGGTTGTAGAGGGCAAGGAC -3'. Human TGFβ2 cDNA was amplified using the forward primer, 5'-GTGAATGGCTCTCCTTCGAC-3', and the reverse primer: 5'-CCTCGAGCTCTTCGCTTTTA-3'. Human β-actin was amplified by using the forward primer, 5'-GGCTGTATTCCCCTCCATCG-3', and the reverse primer: 5'-CCAGTTGGTAACAATGCCATGT-3'.

8. Clonogenic assay

A375 and HPAC cells were plated in six-well plates at 1×10^5 cells/well and infected with adenovirus (Ad-NC, Ad-shTGFβ1, Ad-shTGFβ2). Cells were then trypsinized and plated 48 hr later to 5×10^3 or 1×10^4 cells/well in six-well plates and monitored daily by microscopy. Once cells formed colonies, the plate were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet.

9. Measurement of intracellular level of ROS

Intracellular ROS was assessed using the ROS-specific probe 2'-7'-diclorofluorescein diacetate (DCF-DA, Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated with 20 μM DCF-DA for 1 hr and fluorescence signals were obtained with a fluorescence microscope.

10. Enzyme-linked immunosorbent assay (ELISA)

Cells were plated in six-well plates at 1×10^5 cells/well and supernatants collected 48 hr later to assess the levels of secreted TGF-β1 or TGF-β2 with a commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

11. Immunopricipitation(IP)

Immunopricipitation were performed at 4°C unless otherwise indicated, using a Pierce spin column that can be capped and plugged with a bottom plug for incubation or unplugged to remove the supernatant by centrifugation at 1000 × g for 1 min. Antibody binding to protein A/G agarose was performed as described in the Pierce Crosslink Immunoprecipitation kit with a slight modification. Briefly, protein A/G agarose slurry (20 μL) was washed twice with 200 μL PBS buffer, and then incubated in 10 μL Trx, GSTM1, ASK1, Ap1, Sp1, Smad4 antibody diluted with 90 μL PBS for 30 min at 25°C on a mixer. In parallel, 100 μL of mouse and rabbit serum or anti-mouse and anti-rabbit IgG

peroxidase secondary antibody served as a negative control. The supernatant was subsequently discarded and the beads washed three times with 300 µL PBS, followed by incubation with 50 µL 2.5mM DSS solution at 25°C for 45–60 min on a mixer. The beads were then washed three times with 50 µL 100 mM glycine (pH 2.8), twice with 300 µL 1% NP-40 in PBS, and then once with 300 µL PBS. The antibody-cross-linked beads were incubated overnight at 4°C with 600 µL A375 or HPAC cell lysate pre-cleared with control agarose resin (Pierce, Waltham, MA, USA) for 1 hr on a shaker. After removing supernatant (flow-through) and washing with 300 µL washing buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.4) three times, the immunoprecipitates were eluted with 60 µL Elution buffer and boiled at 100°C for 10 min. The eluate was then subjected to western blotting.

12. Chromatin immunoprecipitation(ChIP) assay

ChIP assays were performed with a kit from Thermo Scientific (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, treated cells were washed with PBS, cross-linked with 1% formaldehyde for 10 min, rinsed with ice-cold PBS, collected into PBS containing protease inhibitors, and then resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris at pH 8.1 with 1% protease inhibitor cocktails). The cells were sonicated to produce 200-1000 bp DNA fragments, and then

centrifuge to remove insoluble material at 9000 × g for 5 min. DNA immunoprecipitation was performed with the indicated antibodies overnight at 4℃. After centrifugation to transfer the supernatant to a new 1.5 mL tube, 20μL beads were added to each IP incubated for 2 hr at 4 ℃, and centrifuged. The beads were washed twice with Wash Buffer 1, once with Wash Buffer 2, once with 150μL 1X IP Elution Buffer, and incubated at 65 ℃ for 30 min, The solutions were then aliquoted into tubes containing NaCl and Proteinase K, incubated at 65 ℃ for 1.5 hr. Subsequently, 750μL of DNA Binding Buffer was added to each tube, mixed, and then 500μL of each sample was transferred to a DNA Clean-Up Column for purification. The resulting DNA was subjected to RT-PCR with primers specific for the human Trx promoter (5'-TCCAGGAGTCTGCCTCTGTTAG-3' and 5'-CTGCTGGA GTCTGACGAGCG-3'), GSTM1 promoter (5'-TAGGATCTGGCTGGTGT CTC-3' and 5'-GTGCGGATTCCGCAGACAGG-3'). PCR reactions were run with Absolute qPCR SYBR Green Fluorescein Mix (Thermo Scientific) with an initial denaturation at 95℃ for 15min, followed by 40cycles of denaturation at 95℃ for 15 s and annealing at 62℃ for 1 min.

13. Animal study

To generate a xenograft tumor model, 8×10^6 A375 and HPAC tumor cells were injected into the subcutaneous abdominal region of male BALB/c athymic

nude mice. When the tumors reached an average size of 60–80 mm^3, the nude mice received intratumoral injections of 1×10^9 plaque forming units (pfu) of one of three defective adenoviruses diluted in 50 μl PBS or PBS alone. The defective adenoviruses used were defective control adenovirus (Ad-NC), TGFβ1/TGFβ2 shRNA-expressing defective adenovirus (Ad-shTGFβ1/TGFβ2. Intratumoral injection was repeated every other day for a total of three injections.

Regression of tumor growth was assessed by taking measurements of the length (L) and width (W) of the tumor. Tumor volume was calculated using the following formula: volume = 0.52 * L * W^2.

14. Immunohistochemistry (IHC)

Tumor tissues were extracted, fixed for 24 hr in 10% formaldehyde, and paraffin embedded for immunohistochemical (IHC) staining. IHC staining was performed as follows. Tissue section slides were deparaffinized twice with xylene for 10 min each and slides were rehydrated using a graded alcohol series. After removing endogenous peroxidases using 0.1% H2O2, slides were washed three times with PBS. Antigen retrieval was performed using 10 mM citrate buffer (pH 6.0) (DAKO, Glostrup, Denmark) and a microwave oven. Tissues were permeabilized with 0.5% PBX (0.5% Triton X-100 in PBS) for 30 min. After blocking for 1 hr with 5% BSA, the primary antibody was added and incubated overnight at 4 ℃. Primary Antibody Enhancer (Thermo Fisher

Scientific, Waltham, MA, USA) and HRP Polymer (Thermo Scientific) were used for signal amplification. To develop the colored product, a mixture of DAB (3,3′-diaminobenzidine) Plus Chromogen and DAB Plus Substrate (Thermo Fisher Scientific) was added for 5 min. After washing with PBS, 20% hematoxylin counterstain was added for 2–5 min to stain the nuclei. Finally, tissue slides were dehydrated in a graded alcohol series. After clearing twice in xylene, tissues slides were coverslipped with mounting media (xylene:mount = 1:1) for microscopy.

15. Statistical analysis

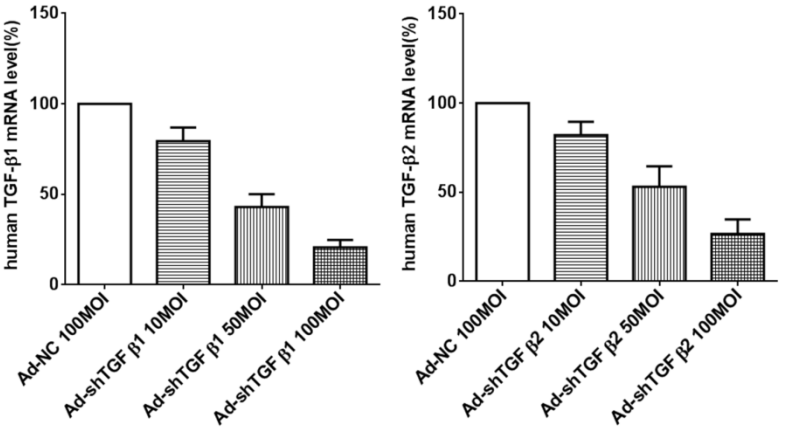
The data were expressed as mean ±standard error (SE). Statistical comparison was made using Graph Pad (Systat Software Inc). P values less than 0.05 were considered statistically significant (*, $P<0.05$; **, $P<0.01$;***, $P<0.001$).

III. RESULTS

1. TGF-β1 or 2 expression in cells after infection with an adenovirus expressing shTGF-β1 or 2

Almost all human tumors overexpress TGF-β, which contributes to the induction of tumor cell invasion and metastasis.[51] Accordingly, in this study shRNAs against TGF-β were used as therapeutic agents. It has been known that TGF-β1 and TGF-β2 are highly expressed in cancer cells, whereas TGF-β3 is rarely expressed. To decrease the expression of TGF-β1 or TGF-β2 protein, recombinant adenoviruses were constructed containing the shRNA of TGF-β1 or TGF-β2. The infection efficiency of adenovirus type 5(AAV5) was examined in human cells before confirming the repression of TGF-β1/2 mRNA and protein. We then examined the knockdown efficacy of adenoviruses expressing shTGF-β1 or 2 with various MOIs (10, 50 and 100) in human A375 cells using RT-PCR and ELISA assays were subsequently performed to determine whether the viruses decreased TGF-β1 or 2 expression at the mRNA or protein level. TGF-β1 mRNA was decreased by 20% at only 10 MOI and TGF-β1 mRNA was suppressed by 80% at 100 MOI, also TGF-β2 mRNA was decreased by 20% at only 10 MOI and TGF-β2 mRNA was suppressed by 75% at 100 MOI in A375 cells (Figure 1A), suggesting that the knockdown efficacy correlated with viral MOI. Similar results were also observed with TGF-β1 or 2 protein expression (Figure 1B).

(A)



(B)

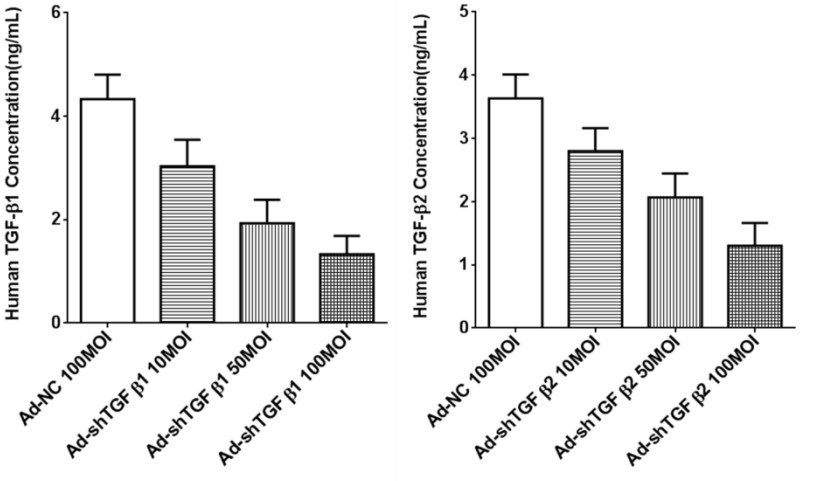


Figure 1. Downregulation of human transforming growth factor (hTGF)-β1 or 2 short hairpin RNA(shRNA). Cancer cells (human A375 cells) were infected with adenovirus-expressing shRNA targeting human TGF-β1(Ad-shTGF-β1), human TGF-β2(Ad-shTGF-β2) or scrambled DNA (Ad-NC). TGF-β1 or 2 mRNA(A) and protein levels(B) were assayed by

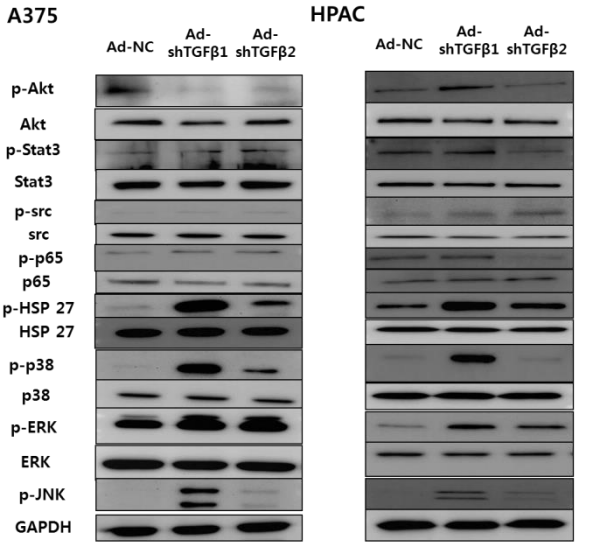
quantitative real-time polymerase chain reaction(qRT-PCR) and enzyme-linked immunosorbent assay(ELISA), respectively. MOI, multiplicity of infection; NC, negative control.

2. Adenovirus expressing shRNA of TGF-β1 or 2 can induce several signaling pathways change in melanoma and pancreatic cancer cell lines

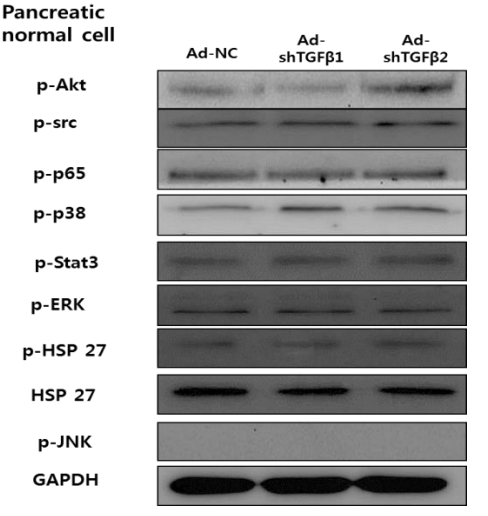
TGF-β is a key signaling molecule overexpressed by many cancers. Therefore, we examined the effect of TGF-β downregulation on various pathways in A375 and HPAC cell lines including p38, HSP27, p65, Src, Akt, Stat3, JNK, Smad and ERK by western blotting. Notably, we observed that activity of phospho-p38 (p-p38) and phospho-JNK were increased by Adenovirus expressing shRNA of TGF-β1 or 2 infection. Also the survival molecules phospho-Akt, phosphor-Src, phospho-p65, phospho-stat3 were decreased (Figure 2A). However, in pancreatic normal cells the various key signaling pathway molecules, including p38, HSP27, p65, Src, Akt, Stat3, JNK, Smad and ERK expression were not changed (Figure 2B). Next, in order to examine whether shTGFβ1 or 2 adenovirus inhibits cancer cell survival and proliferation, MTT assays and clonogenic assays were performed, which can measure short-term and long-term cancer cell survival. The MTT assay results showed that shTGFβ1 or 2 adenovirus infection in A375, HPAC cells induced significant reduction of cell survival (Figure 2C). The clonogenic assay results indicated that clonogenic assay survival decreased in both melanoma and

pancreatic cancer cells when infected with shTGFβ1 or 2 adenovirus (Figure 2D).

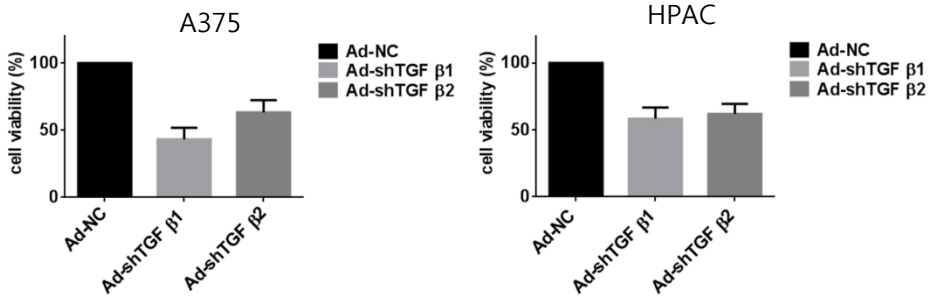
(A)



(B)



(C)



(D)

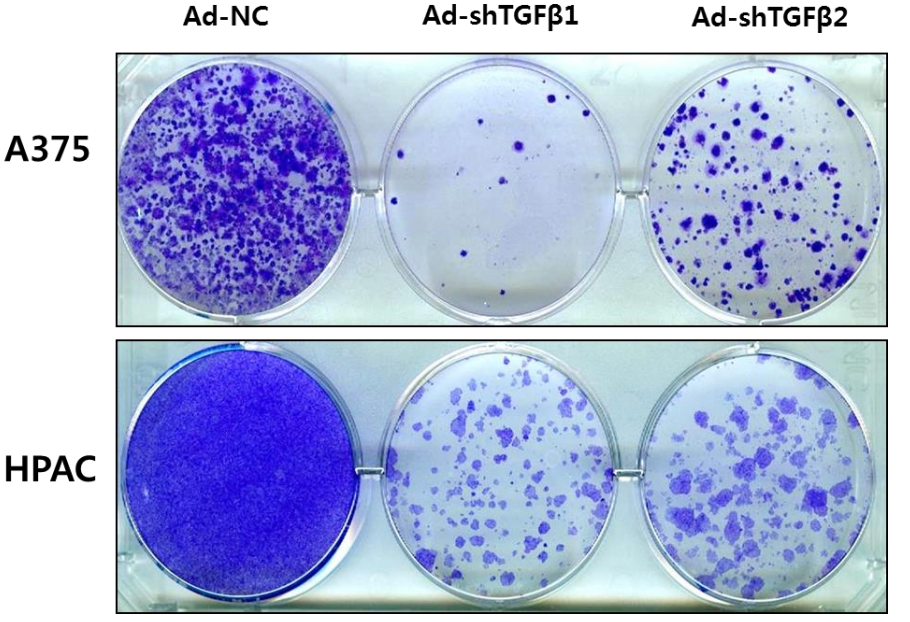


Figure 2. Effect of adenovirus-expressing shTGF-β1 or 2 in melanoma and pancreatic cancer cell lines. (A) A375 and HPAC cell lines were treated using adenovirus-expressing shTGF-β1/2 at 100MOI respectively. After 48 hr, the expression of p-p38, p38, p-HSP27, HSP27, p-ERK, p-Src, p-p65, p-JNK, p-stat3 and GAPDH were detected via western blot analysis. (B) Pancreatic

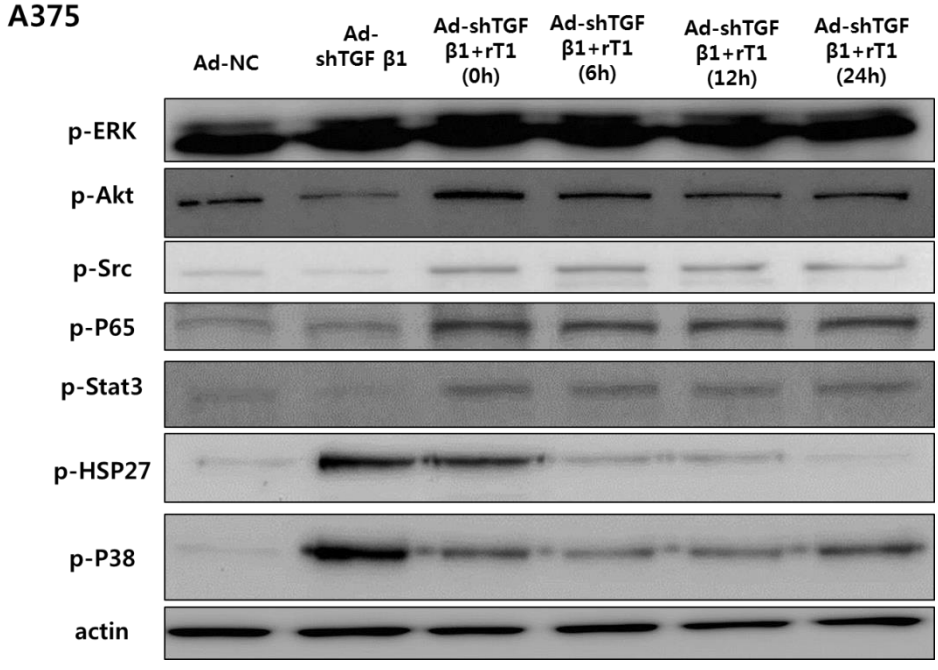
normal cell lines were treated using adenovirus-expressing shTGF-β1/2 at 100MOI respectively. After 48 hr, the expression of p-p38, p-Akt, p-HSP27, HSP27, p-ERK, p-Src, p-p65, p-JNK, p-stat3 and GAPDH were detected via western blot analysis. (C) A375 and HPAC cells were treated with adenovirus-expressing shTGF-β1/2. After 48 hr, cell viability was tested via a MTS viability assay. Error bars represent the standard error from three independent experiments. (D) A375 and HPAC cells were treated with adenovirus-expressing shTGF-β1/2 for 48 hr, and incubated for an additional 14 days for clonogenic assays.

3. No off-targeting effect of adenovirus-expressing shTGF-β1 or 2 in melanoma cancer cell lines

Despite the recent advances in gene-engineering technologies.[52] RNAi remains one of the most versatile and powerful tools to stydy and manipulate gene expression in eukaryotic organisms.[53] One specific variant is shRNA that consists of a stem composed of an antisense (or guide) strand that is complementary to a target mRNA and a sense (or passenger) strand that ideally is inert and merely provides structure to complete the double-stranded molecule. In addition to binding their designated target, shRNA antisense strands can also recognize and degrade other mRNAs with similar complementarity, resulting in “off-target” effects. In order to find out if there have off-targeting effects while infection with adenovirus-expressing shTGF-β1 or 2, A375 cells were infected

with one shRNA and examined by western blotting after recombinant TGFβ1 treatment. We can see that the p-Src, p-P65, p-Stat3, p-HSP27, p-P38 expression were clearly recoverd from treatment with recombinant TGFβ1 protein (Figure 3A). Also morphology shows a similar result with western blot data (Figure 3B). Therefore, these results suggest that no off-targeting effects were associated with adenovirus-expressing shTGF-β1 or 2 treatment.

(A)



(B)

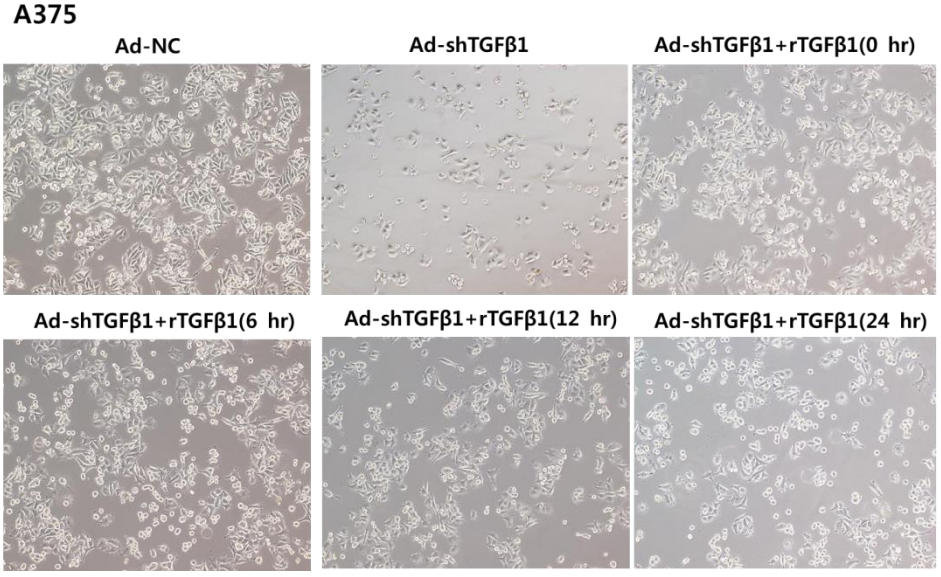
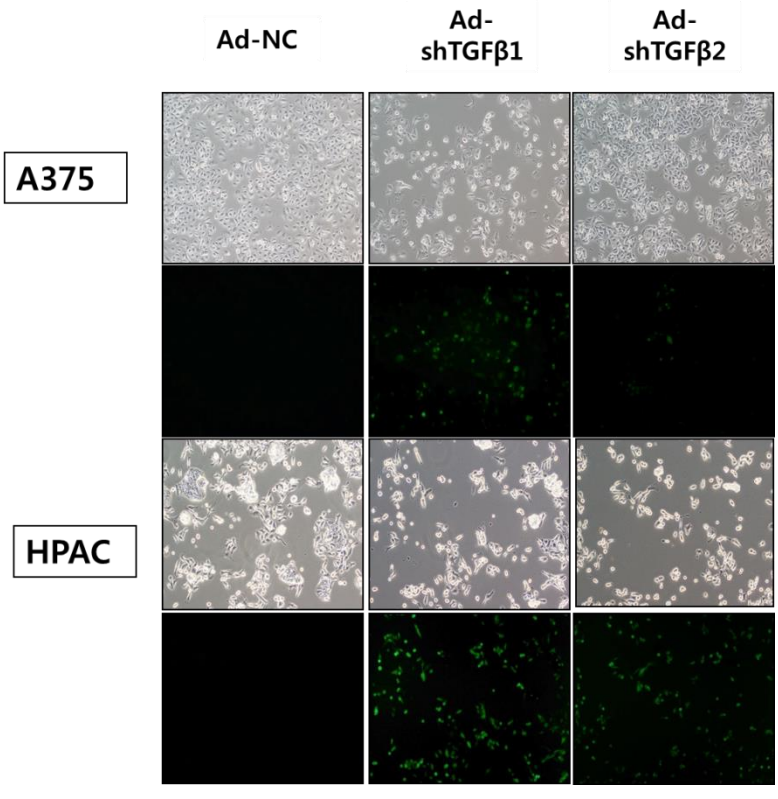


Figure 3. No off-targeting effect of adenovirus-expressing shTGF-β1 or 2 in melanoma cancer cell lines. (A) A375 cell lines were infected with adenovirus-expressing shTGF-β1 at 100MOI with or without of recombinant TGF-β1 750ng/ml for time-dependent, respectively. After 48 hr, the expression of p-p38, p-Akt, p-HSP27, p-ERK, p-Src, p-p65, p-stat3 and GAPDH were detected via western blot analysis. (B) A375 cell lines were infected with adenovirus-expressing shTGF-β1 at 100MOI with or without of recombinant TGF-β1 750ng/ml for time-dependent, respectively. After 48 hr, morphological changes were observed by using microscopy.

4. Increased ROS generation was induced by adenovirus expressing shRNA of TGF-β1 or 2

ROS has been reported to be related to JNK and p38 pathways in many studies.[54] Thus, we assessed ROS generation by adenovirus expressing shRNA of TGF-β1 or 2 infection. As a result, ROS was increasingly generated after 48 hr of adenovirus expressing shRNA of TGF-β1 or 2 infection in A375, HPAC cells (Figure 4A). whereas little amount of ROS generation was observed in pancreatic normal cells (Figure 4B).

(A)



(B)

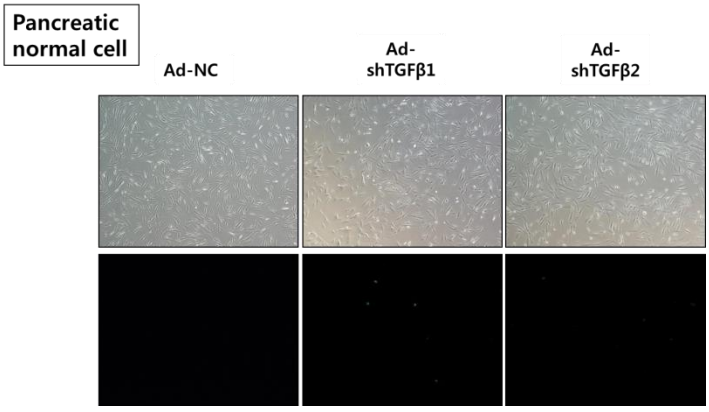


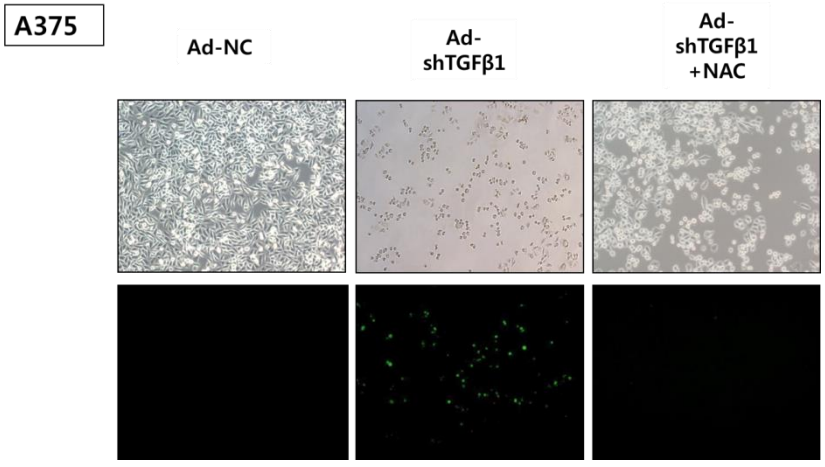
Figure 4. ROS generation was induced by shTGF-β1 and shTGF-β2 expressing adenoviruses. (A) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI, respectively after 48 hr, incubation with DCF-DA (20 μM) for 1 hr. (B) Pancreatic normal cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI respectively after 48 hr, incubation with DCF-DA (20 μM) for 1 hr.

5. Effects of NAC on cell growth and apoptosis in Adenovirus expressing shRNA of TGF-β1 or 2 treated melanoma and pancreatic cancer cells

N-acetylcysteine (NAC) is an aminothiol and synthetic precursor of intracellular cysteine and GSH and a strong antioxidant widely used to investigate the role of ROS in apoptosis.[55] The effect of NAC on cell growth and apoptosis after adenovirus expressing shRNA of TGF-β1 or 2 infection treated melanoma and pancreatic cancer cells were then examined. As a result,

the ROS production was significantly reduced by NAC treatment, however the cell death still remained(Figure 5A, 5B), which suggest that the cell death induced by Adenovirus expressing shRNA of TGF-β1 or 2 treatment were correlated with survival molecule downregulation and ROS produciton.

(A)



(B)

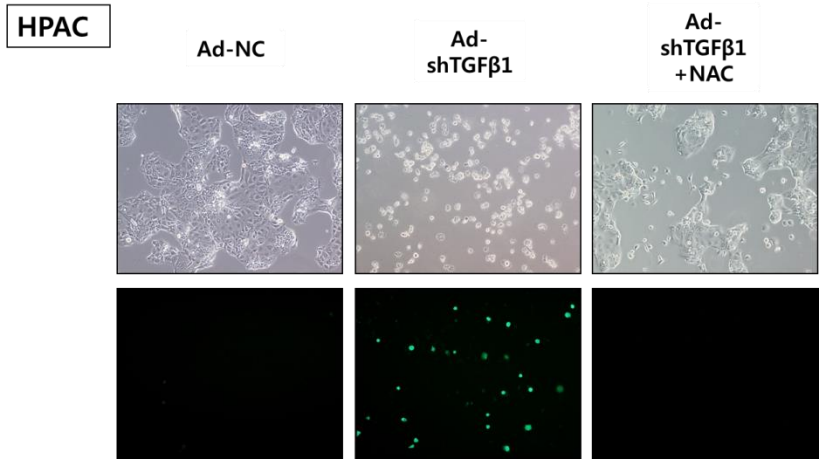


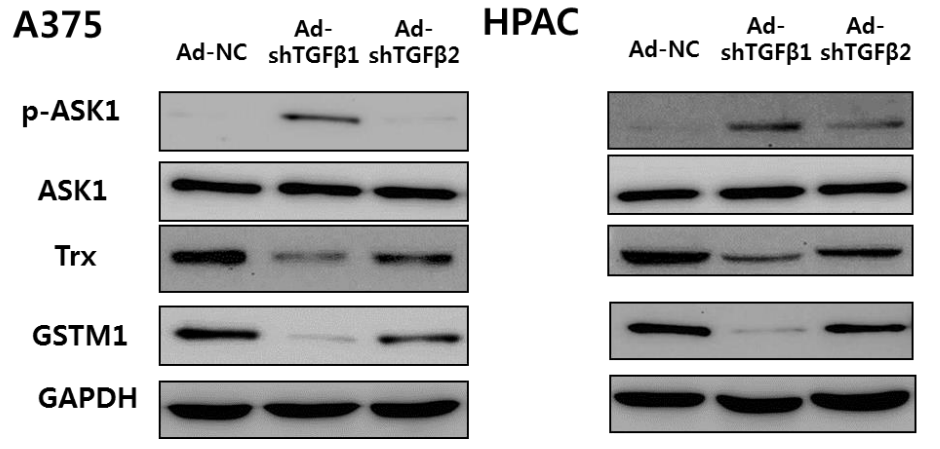
Figure 5. Effects of NAC treatment with Adenovirus expressing shRNA of TGF-β1 or 2 in melanoma and pancreatic cancer cells. (A) A375 cell lines

were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 6 hr, infected cells were treated with NAC (10 mM) for 42 hr, then incubated with DCF-DA (20 μM) for 1 hr. (B) HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 6 hr, infected cells were treated with NAC (10 mM) for 42 hr, then incubated with DCF-DA (20 μM) for 1 hr.

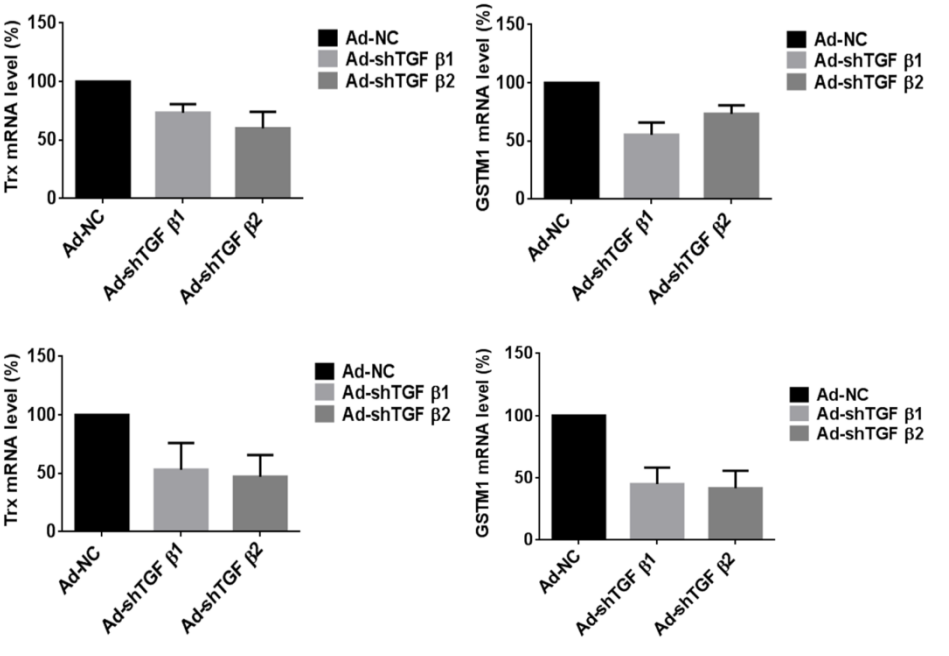
6. Dissociation of Trx from ASK1–Trx complexes induced by Adenovirus expressing shRNA of TGF-β1 or 2 infection

Trx and GSTM1 have been identified as binding proteins of ASK1. Trx has been shown to inhibit signal cascades downstream of ASK1 in a redox-dependent manner.[56] ROS such as hydrogen peroxide which was produced by adenovirus expressing shRNA of TGF-β1 or 2 infection is able to dissociate Trx or GSTM1 from ASK1. Both cellular protein levels of Trx and GSTM1 expression (Figure 6A) and mRNA level of Trx and GSTM1 expression (Figure 6B) were decreased by shTGFβ1 or 2 adenovirus infection. Moreover, interaction between endogenous Trx and ASK1 (Figure 6C) or interaction between endogenous GSTM1 and ASK1 (Figure 6D) was decreased by adenovirus expressing shRNA of TGF-β1 or 2 infection. These results suggest that the increased ASK1 activity was correlated with both of reduction of Trx and GSTM1 expression and dissociation of Trx and GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes, respectively.

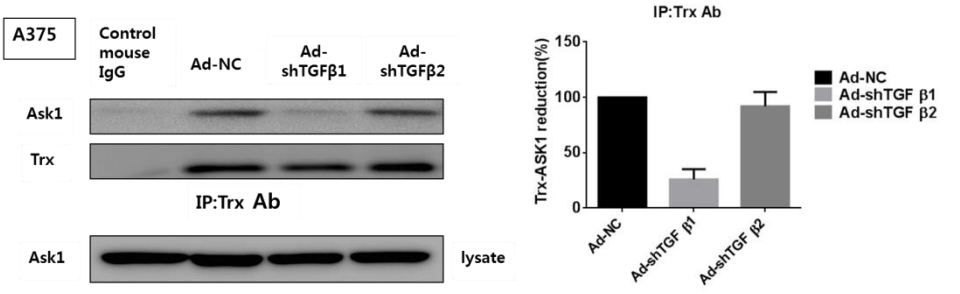
(A)



(B)



(C)



(D)

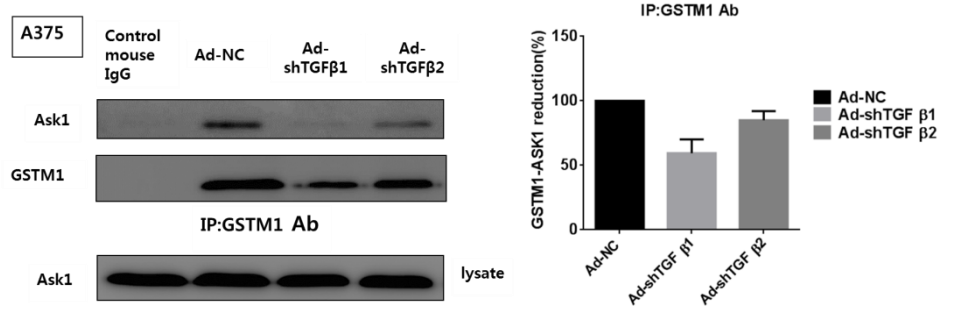


Figure 6. Effects of NAC treatment with Adenovirus expressing shRNA of TGF-β1 or 2 in melanoma and pancreatic cancer cells. (A) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, the expression of p-ASK1, ASK1, Trx, GSTM1 and GAPDH were detected by western blot analysis. (B) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, the expressions of Trx and GSTM1 mRNA level were assayed by quantitative real-time polymerase chain reaction (qRT-PCR). (C) A375 cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, lysates were then subjected to

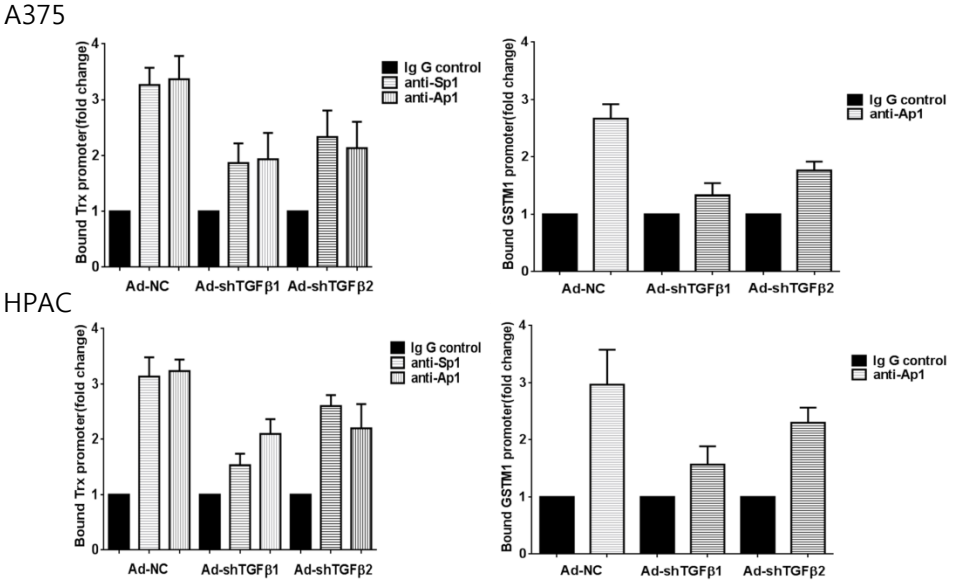
immunoprecipitation with using an anti-Trx antibody to identify changes in complex formation. (D) A375 cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, lysates were then subjected to immunoprecipitation with using an anti-GSTM1 antibody to identify changes in complex formation.

7. Regulation of Trx, GSTM1 promoter activity, Ap1, Sp1 and Smad molecule expression by TGFβ

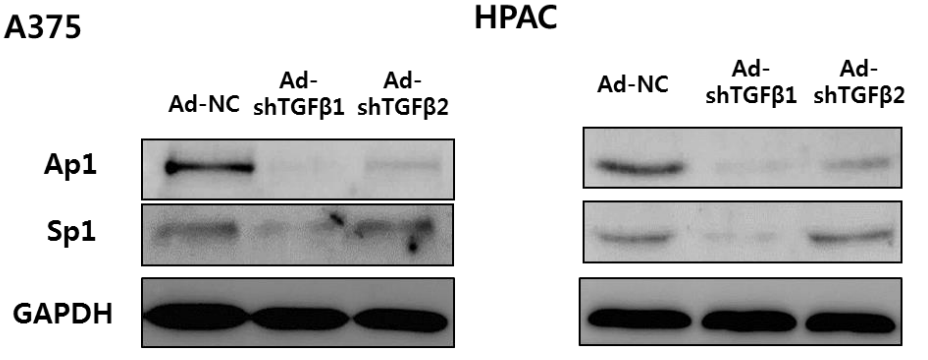
While, Trx promoter contains several consensus Ap1 and Sp1 binding sites, whereas the GSTM1 promoter contains only Ap1 binding sites. TGFβ regulates gene transcription primarily through the intracellular Smad signaling cascade, which is initiated by binding of the TGFβ ligand to heteromeric complexes of specific type II (TβR-II) and type I (TβR-I) kinase receptors.[57] Treatment with adenovirus expressing shRNA of TGF-β1 or 2, however, Trx and GSTM1 promoter activity were decreased (Figure 7A), and the Ap1 and Sp1 protein levels were also reduced by adenovirus expressing shRNA of TGF-β1 or 2 infection (Figure 7B). SMADs are intracellular proteins that transduce extracellular signals from TGF β ligands to the nucleus where they activate downstream gene transcription.[57] While infection with adenovirus expressing shRNA of TGF-β1 or 2, the expression of p-Smad2 and p-Smad3 were decreased (Figure 7C). Also, the physical interaction between Ap1 or Sp1 and the Smad proteins were decreased (Figure 7D). This suggests that decreased

level of Sp1, Ap1 gene expression and reduction of the interaction between Ap1 or Sp1 and the Smad proteins played a causative role for the reduction of Trx and GSTM1 gene expression.

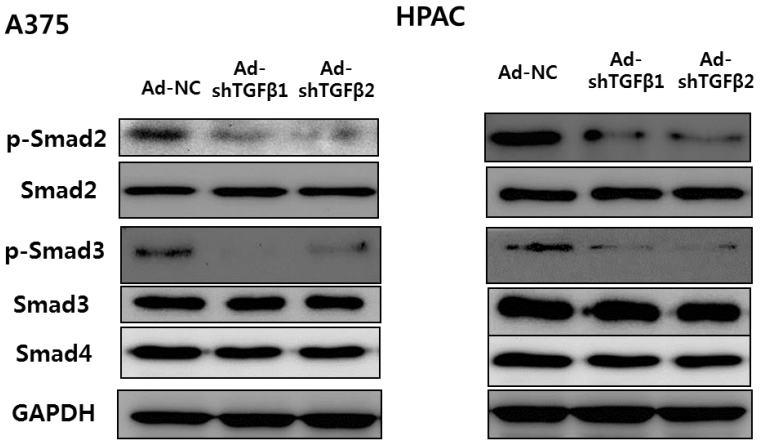
(A)



(B)



(C)



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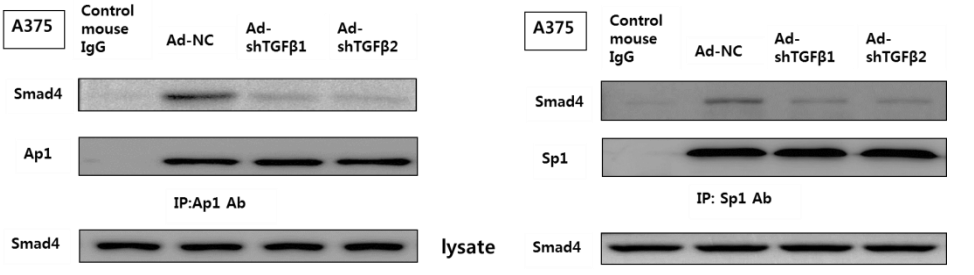


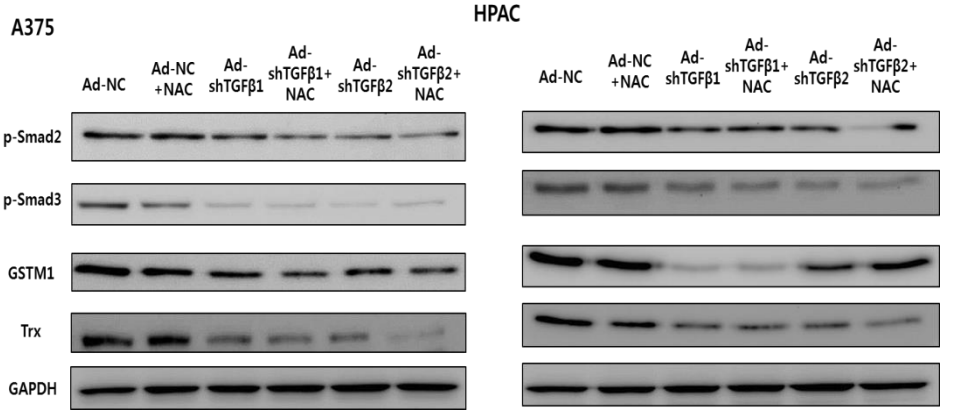
Figure 7. Regulation of Trx, GSTM1 promoter activity by TGFβ. (A) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, Trx, GSTM1 promoter activity were analysed by Chip assays. (B) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, the expression of Ap1, Sp1 and GAPDH were detected by western blot analysis. (C) Also the p-Smad2, Smad2, p-Smad3, Smad3, Smad4 and GAPDH were detected by western blot analysis. (D) A375 cell lines were infected with

adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 48 hr, lysates were then subjected to immunoprecipitation with using an anti-Ap1 and anti-Sp1 antibody to identify changes in complex formation.

8. No effect of expressing shRNA of TGF-β1 or 2 adenovirus treatment induced ROS on Trx, GSTM1 expression

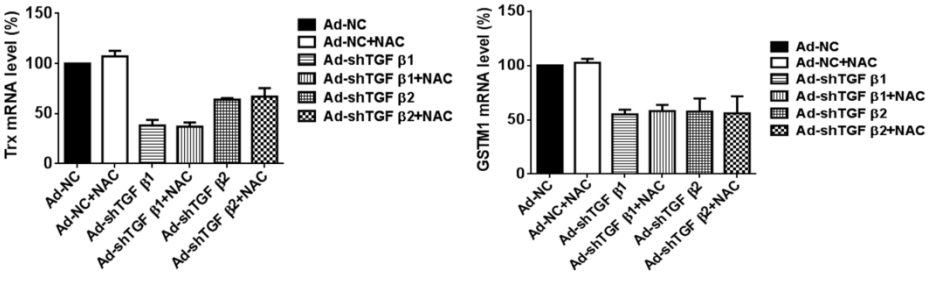
Even though ROS can dissociate Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes, it remains unclear whether it also regulates Trx and GSTM1 gene expression. We assessed the impact of ROS on Trx and GSTM1 expression. NAC was combined with adenovirus expressing shRNA of TGF-β1 or 2. Interestingly, Trx and GSTM1 expressions were not likely to be changed much by NAC (Figure 8A, B, C). This suggests that ROS is involved in the dissociation of Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes, but not Trx and GSTM1 gene expression.

(A)



(B)

A375



(C)

HPAC

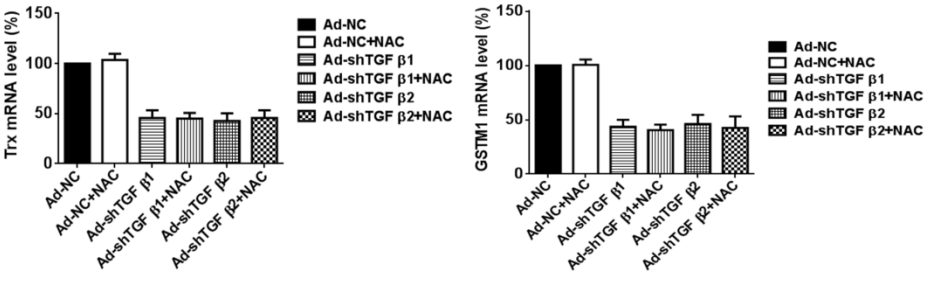


Figure 8. Effects of NAC on Trx, GSTM1 expression with expressing shhTGF-β1 or 2 adenovirus infection in melanoma and pancreatic cancer cells. (A) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 6 hr, infected cells were treated with NAC (10 mM) for 42 hr. Then the p-Smad2, p-Smad3, Trx, GSTM1 and GAPDH were detected by western blot analysis. (B), (C) A375 and HPAC cell lines were infected with adenovirus-expressing shTGF-β1 or 2 at 100 MOI, respectively. After 6 hr, infected cells were treated with NAC (10 mM) for 42 hr. Then the expressions of Trx and GSTM1 mRNA level were assayed by

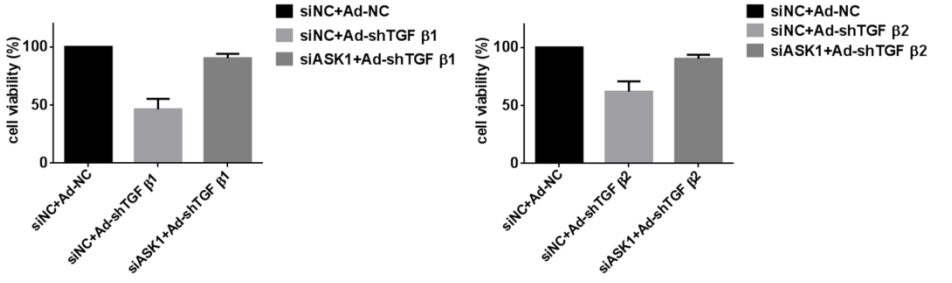
quantitative real-time polymerase chain reaction (qRT-PCR).

9. ASK1 mediates TGFβ induced cell death via p38 MAPK/JNK activation

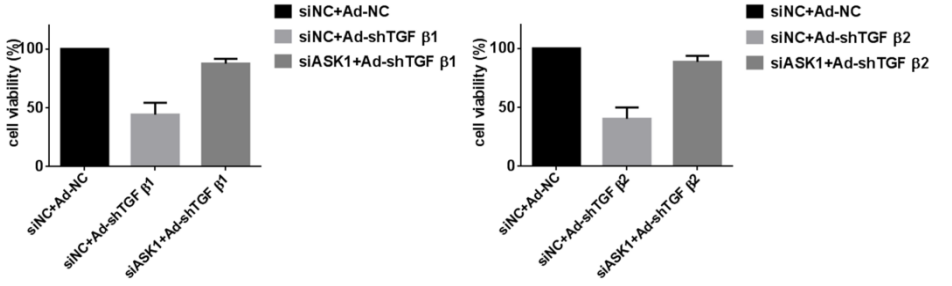
As MAPKs are activated during adenovirus-expressing shTGF-β1 or 2-induced apoptosis of A375 and HPAC cells, we wanted to investigate the possible role of ASK1 in regulating p38 MAPK/JNK activation and whether inhibition of ASK1 activity would result in a corresponding inhibition of cell death. A375 and HPAC cells stably transfected with siASK1 were used, and combined with infection of adenovirus-expressing shTGF-β1 or 2. As shown in figure 9A, downregulation of ASK1 with siASK1 increased the viability of adenovirus-expressing shTGF-β1 or 2-infected cells, and the morphology of cells undergoing cell death seemed to be recovered (Figure 9B, 9C). The p38 MAPK/JNK activation were also inhibited by siASK1 (Figure 9D). Futher, overexpression of a dominant-negative kinase-inactive mutant of ASK1(ASK1 -KM) shows an identical pattern to that of siASK1(Figure 9E, 9F, 9G, 9H), suggesting that ASK1 signaling cascade via p38 MAPK/JNK activation were likely the main pathway of adenovirus-expressing shTGF-β1 or 2-induced cell death.

(A)

A375



HPAC



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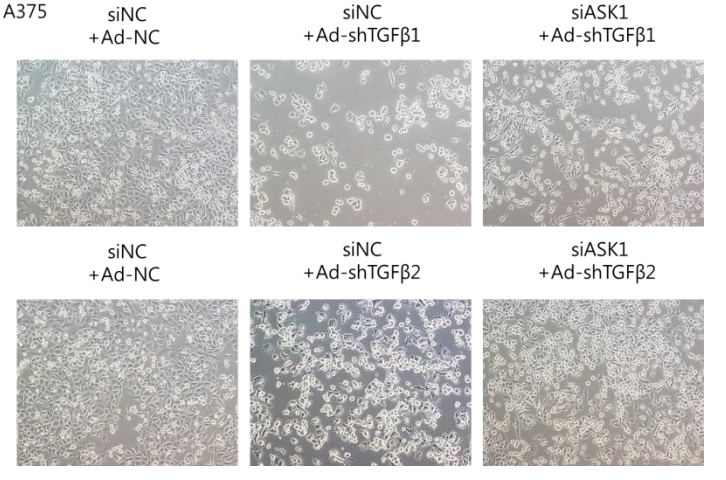
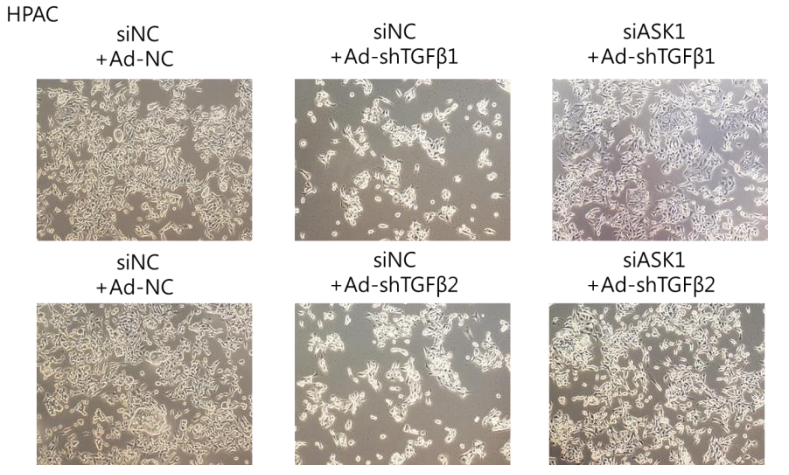
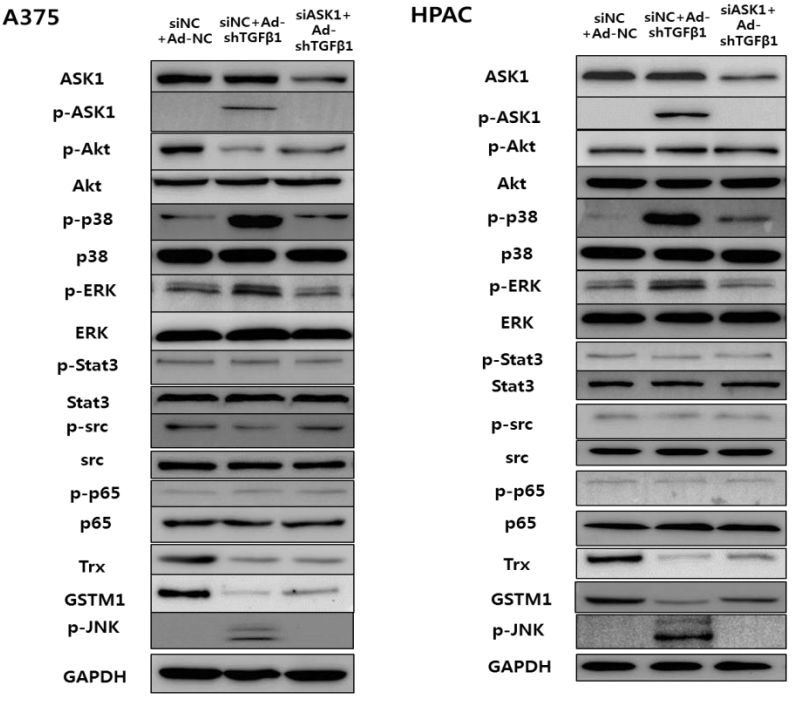


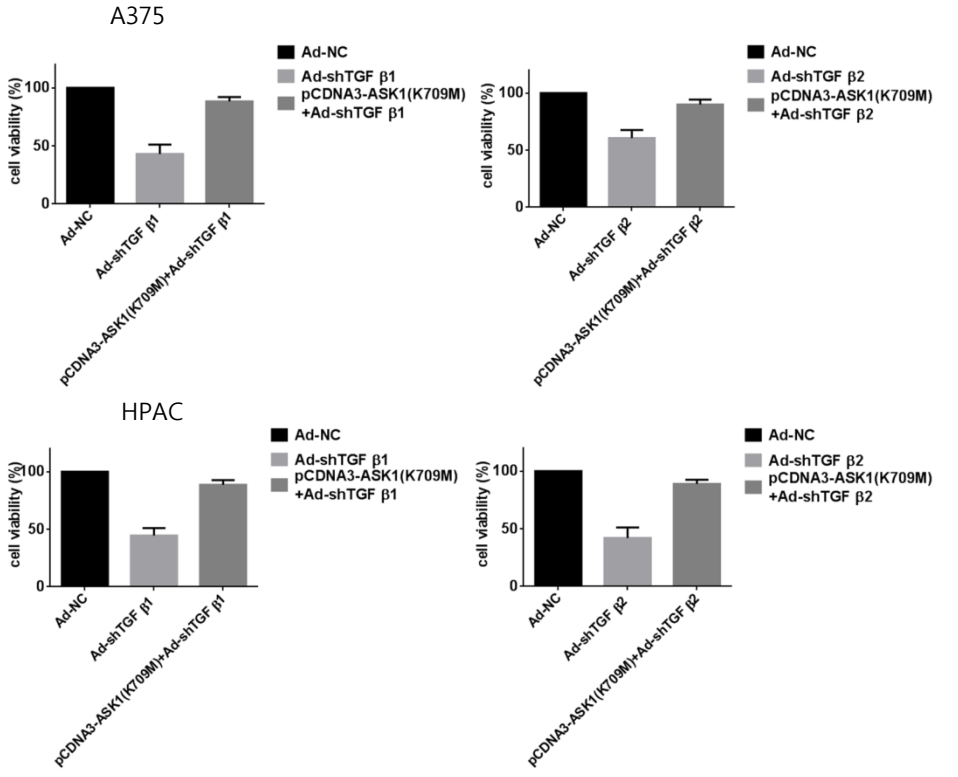
(C)



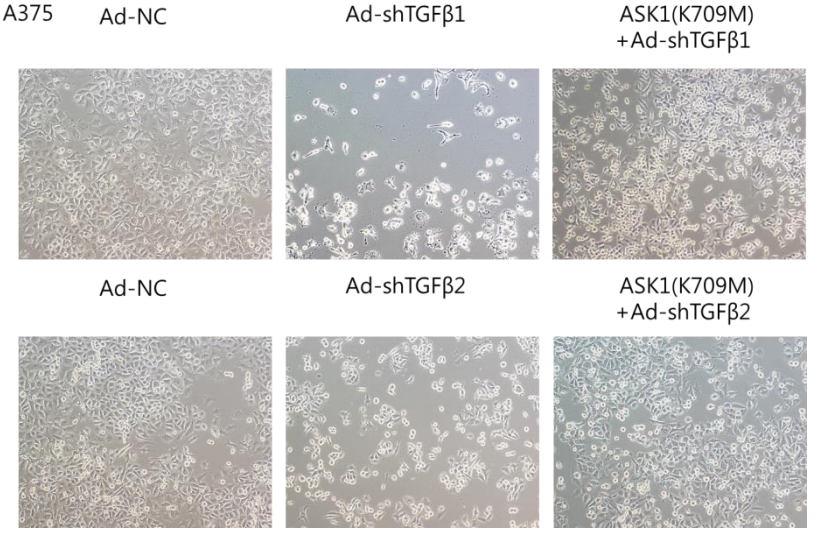
(D)



(E)



(F)



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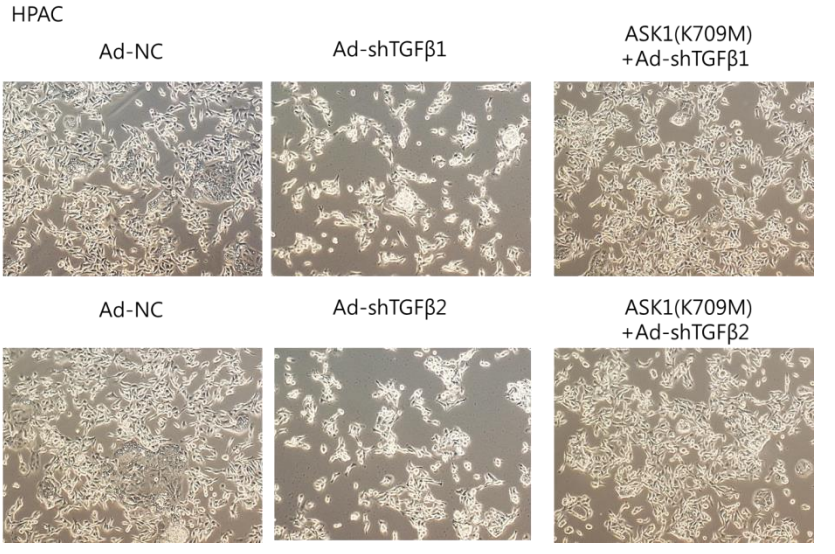

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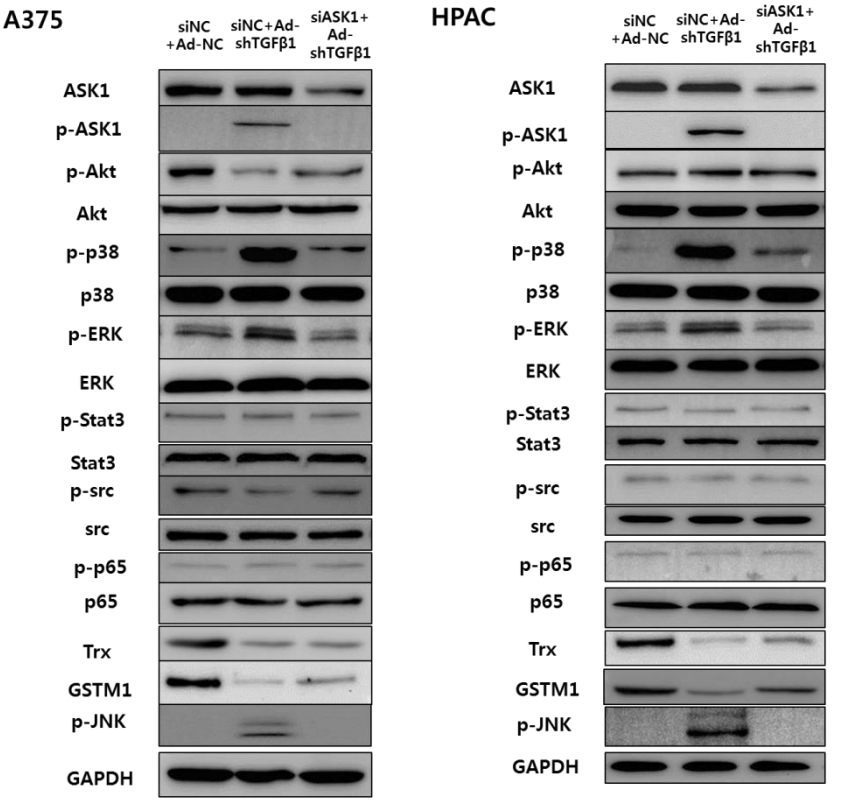


Figure 9. ASK1 mediates TGFβ induced cell death via p38 MAPK/JNK activation. (A) A375 and HPAC cells were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI and transfected with siASK1 (200nM) subsequently. After 48 hr, cell viability was tested by an MTS viability assay. Error bars represent the standard error from three independent experiments. (B), (C) A375 and HPAC cells were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI and transfected with siASK1 (200nM) subsequently. After 48 hr, morphological changes were observed using microscopy. (D) A375 and HPAC cells were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI and transfected with siASK1 (200nM) subsequently. After 48 hr, the expression of p-p38, p-Akt, p-HSP27, HSP27, p-ERK, p-Src, p-p65, p-JNK, p-stat3 and GAPDH were detected by western blot analysis. (E) A375 and HPAC cells were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI and transfected with ASK1-KM 1μg subsequently. After 48 hr, cell viability was tested by an MTS viability assay. Error bars represent the standard error from three independent experiments. (F), (G) A375 and HPAC cells were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI and transfected with ASK1-KM 1μg subsequently. After 48 hr, morphological changes were observed using microscopy. (H) A375 and HPAC cells were infected with adenovirus-expressing shTGF-β1 or 2 at 100MOI and transfected with ASK1-KM 1μg subsequently. After 48 hr, the expression of p-p38, p-Akt, p-HSP27, HSP27, p-ERK, p-Src, p-p65, p-JNK, p-stat3 and GAPDH were

detected by western blot analysis.

10. Enhanced anti-tumor effect induced by adenovirus expressing shTGF-β1 or 2

After a series of in vitro experiments, we confirmed that ASK1 mediated p38 MAPK/JNK activation was likely responsible for adenovirus-expressing shTGF-β1 or 2-induced cell death. Subsequently, we designed an in vivo experiment in xenograft animal models to confirm the anti-tumor effect of adenovirus-expressing shTGF-β1 or 2.

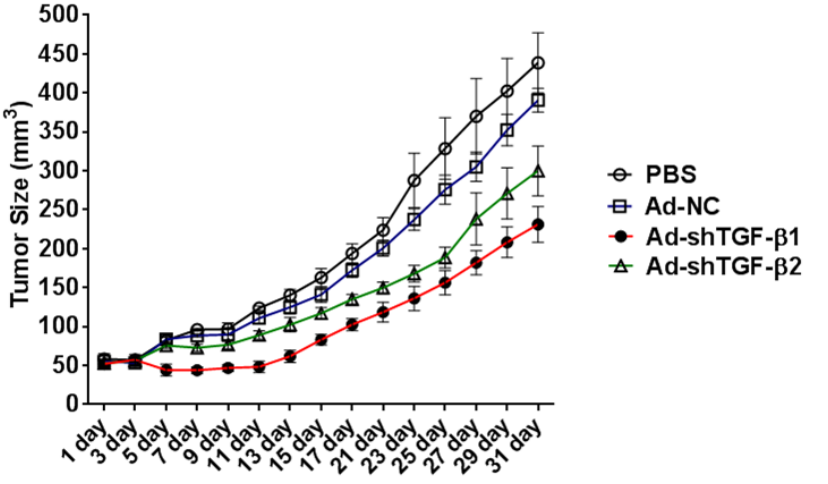
Our results showed that treatment with adenovirus-expressing shTGF-β1 or 2 increased anti-tumor abilities in comparison to PBS or negative control, and TGF-β1 downregulation was better in tumor regression than TGF-β2 downregulation (Figure 10A).

The result of immunohistochemical analysis showed that TGF-β1 or 2 expression was reduced by treatment with adenovirus-expressing shTGF-β1 or 2 compared with PBS and NC virus treated tumor tissues (Figure 10B).

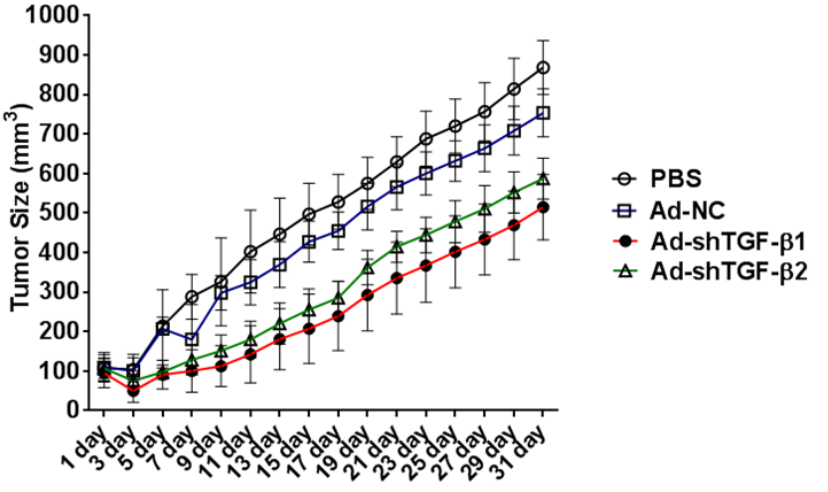
As shown in ex vivo experiments, we confirmed that adenovirus-expressing shTGF-β1 or 2 treatment could increase the anti-tumor effect. Therefore, TGF-β in various tumor cells could be an attractive target for the anti-tumor therapy.

(A)

A375



HPAC



(B)

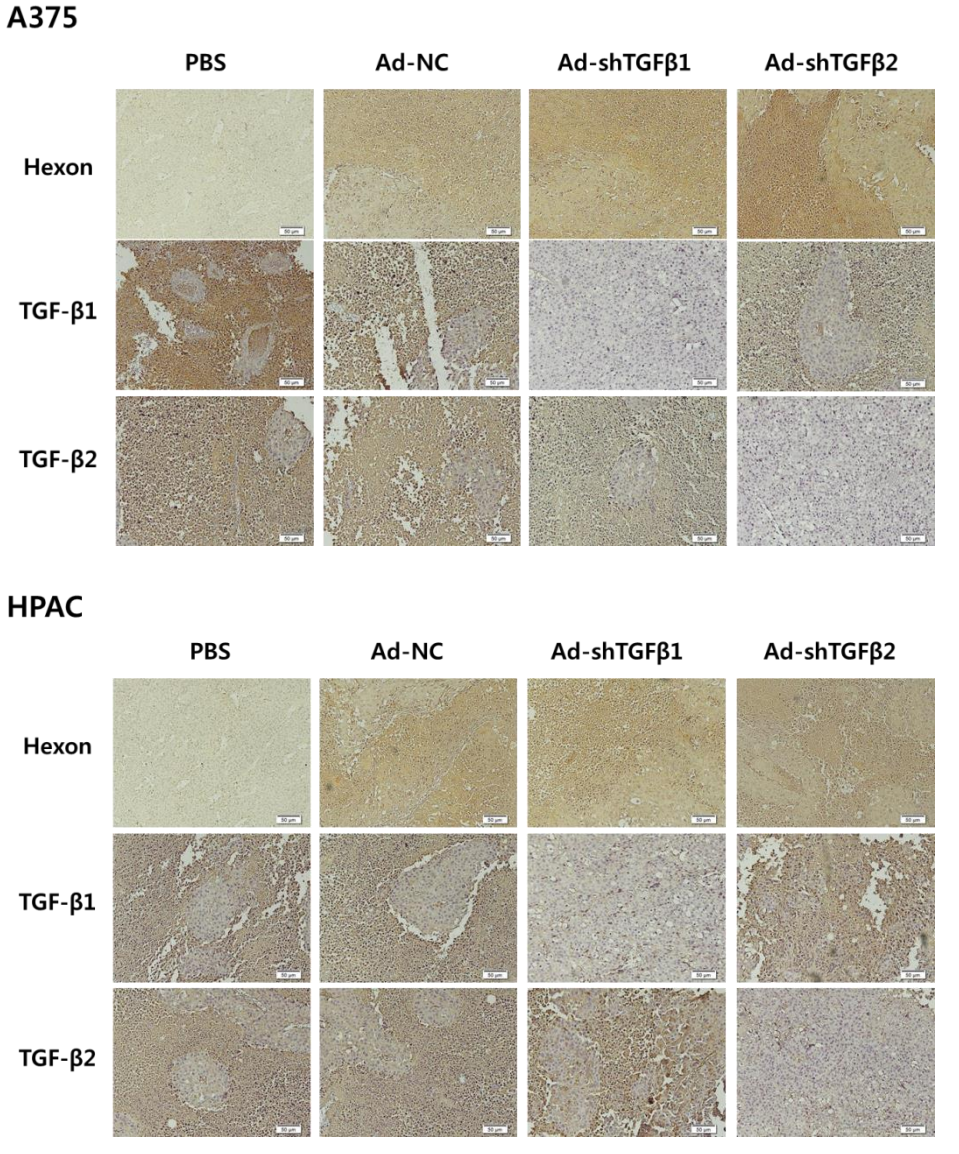


Figure 10. Anti-tumor effect of adenovirus expressing shTGFβ1 or 2 in Xenograft animal models. (A) BALB/c nude mice were injected with 7×10^6 cells/100 μL of A375 and HPAC cells. Seven days after the injection of tumor

cells, BALB/c nude tumor-bearing mice were treated with intratumoral injections of 1 × 10^9 PFU/50 μL of PBS, Ad-NC, Ad- shTGF-β1, Ad-shTGF-β2 virus every other day for a total of 3 injection. (B) Tumors were collected day 11 for histological analysis. Paraffin-embedded sections of tumor tissue were stained with anti-adenovirus type 5(top row, original magnification: ×200), anti-TGF-β1 (second row, original magnification: ×200), and anti-TGF-β2 (third row, original magnification: ×200) antibodies.

IV. DISCUSSION

In many cancers, the expression of TGF-β isoforms were increased. For example, high levels of TGF-β1 have been detected in the gastric cancer patients.[58] The expression levels of TGF-β1 and TGF-β2 are also increased markedly in hepatocellular carcinoma (HCC).[59] Overexpression of TGF-β2 in cholangiocarcinoma promotes tumor cell proliferation.[60] In addition, the overexpression of TGF-β contributes significantly to the development of pancreatic cancer.[61] These results suggest that the major active isoform of TGF-β may be different depending on cancer cell types. In this study, shRNA expressed in viral vector was used to suppress the expression of TGF-β1 or TGF-β2. A strong inhibition of tumor growth and survival was expected to follow the suppression of TGF-β1 or TGF-β2. Then, we showed the effect of shRNA of TGF-β1 was stronger than that of shRNA of TGF-β2 (Figure 2, 6, 7). When the expression of TGF-β1 or TGF-β2 was decreased, ROS generation was increased and the patterns of signaling molecules were changed. And sequentially, Trx and GSTM1 gene expression were decreased, and dissociation of Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes were increased. Intriguingly, from these results, we found that ASK1 activation induced by TGF-β downregulation was proceeded by two different separate pathways: One is through decreased gene expression of ASK1-inhibitory binding proteins, and the other is through ROS generation for the dissociation of ASK1-inhibitory

binding proteins. However, the underlying mechanism of how TGF-β downregulation could induce ROS generation was not yet fully understood.

Under the context of physiological/pathophusiological settings, MAPKs are of vital importance to the life or death for a cell.[62] It is proved that the activation of p38 MAPK/JNK results in apoptosis.[63] As is mentioned before, ASK1 belongs to the mitogen-activated protein kinase kinase kinase family, which is sensitive to different stimuli.[64] A TGF-β protein touches a receptor on the cell surface, which directs some relevant SMAD protein to activate, marks the start of signaling process. A protein complex comes into existence when SMAD proteins attaches to the SMAD4 protein. After the combination, the complex transfer to cell nucleus, in which it binds to specific areas of DNA, such as Trx promoter region of Ap1 and Sp1, and then regulate this genes expression. However, silencing TGFβ1 or 2 can reduce this interaction, also decrese the Ap1 and Sp1 expression, so that the Trx expression was decreased (Figure 7). These results suggest that ASK1 signaling cascade via p38 MAPK/JNK activation were likely the main pathway of adenovirus-expressing shTGF-β1 or 2-induced cell death. While with the downregulation of TGF-β1, p-Akt expression was decreased. As is well known that Akt was involved in cellular survival pathways, by inhibiting apoptotic processes. TAK1 is a member of the MAPKKK family and is activated by various cytokines, including TGF-β family ligands.[65, 66] Several recent stydies show that TAK1 activation is required to induce Akt activation, and the inhibition of TAK1 reduces the activation of

Akt kinase.[67] Unfortunately, however, the influence for cell death by reduction of Akt expression after downregulation of TGF-β1 needs to be further elucidated.

Taken all together, we demonstrate that treatment with adenovirus expressing shRNA of TGF-β1 or 2 can cause various cell death, which was caused by ASK1 activation followed by p38 and JNK activation. ASK1 activation was also related to the reduction of Trx and GSTM1 gene expression and dissociation of Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes. And the effect of shRNA of TGF-β1 was stronger than that of TGF-β2 (Figure 11).

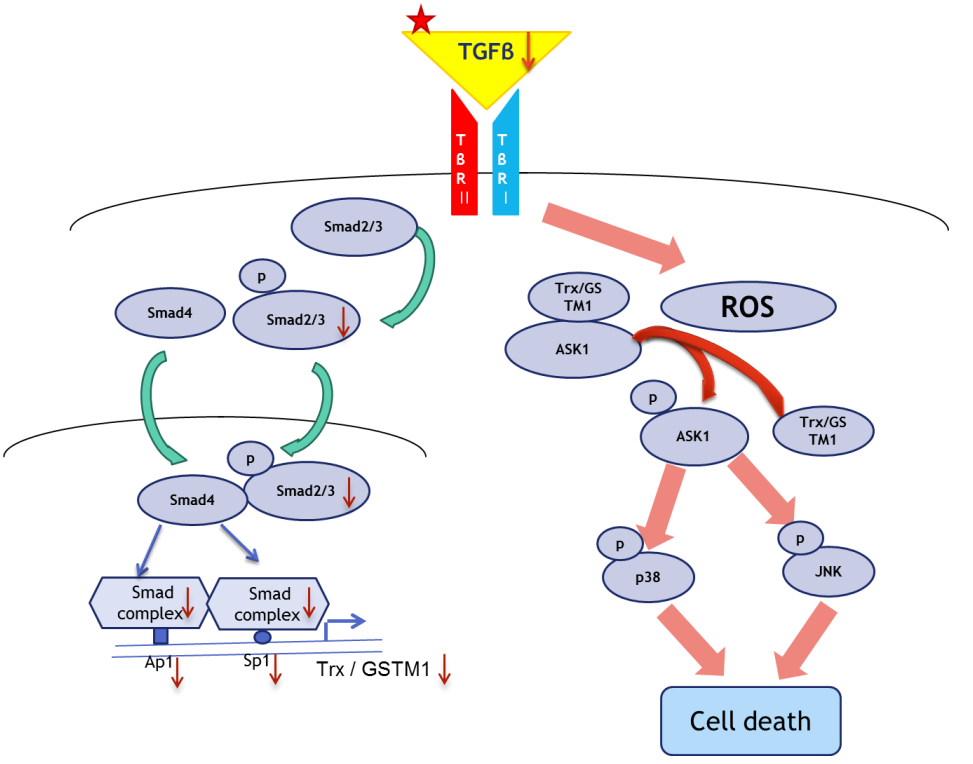


Figure 11. Schematic diagram of determination of cancer cell death by TGF-β downregulation.

TGF-β1 or 2 downregulation can cause both ROS production and reduction of Smad complex (phospho-Smad2, 3 with Smad4) that translocates to the nucleus to bind to gene promoters. ROS can dissociate Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes, which induces ASK1 activation. ASK1 activation is also related to the reduction of Trx and GSTM1 gene expression, which results from decreased transcriptional activity of Smad complex. Conclusively, cancer cell death is caused by ASK1 activation followed by p38 and JNK activation.

V. CONCLUSION

In this study, we demonstrated that TGF-β downregulation induced by adenovirus infection expressing shRNA of TGF-β1 or 2 could cause tunmor cell death, which was mediated by ASK1 activation via p38 and JNK activation. ASK1 activation was also related to both of reduction of Trx and GSTM1 gene expression and dissociation of Trx or GSTM1 from ASK1–Trx, ASK1-GSTM1 complexes. Finally, it was demonstrated that TGF-β in various tumor cells could be an attractive target for the anti-tumor therapy.

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ABSTRACT (IN KOREAN)

종양세포에서 TGFβ1, TGFβ2 isotype의 downregulation에 의한 차등적 세포사멸 기작연구

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한 철 수

TGF-β 신호전달체계는 암의 핵심 동력으로 인식되고 있다. 정상 조직에서의 종양 억제 기능과 달리 TGF-β 활성화는 암 조직에서 종양 촉진을 유발한다. TGF-β의 발현 증가는 종종 많은 악성 종양과 관련이 있다.

본 연구에서는 human transforming growth factor-β (TGF-β)1 또는 2 유전자를 타겟팅하는 short hairpin RNA를 발현하는 유전자조각을 삽입한 아데노바이러스를 이용하여 암세포사멸기전을 밝혀내려고 하였다. 먼저 각 유전자를 삽입한 아데노바이러스의 항 종양효과를 확인한 결과 p38, c-Jun N-terminal kinases (JNKs)의 활성이 증가되고 Akt, Src, Stat3와 p65의 활성이 감소되는 것을 관찰하였다. p38 와 JNK의 활성이 증가되면서 ASK1의 활성과 활성산소의 생성도 증가되는 것도 관찰하게 되였다. 또한 ASK1에 결합하는 Trx 와 GSTM1의 발현이 감소되고 Trx 와 ASK1, GSTM1 와 ASK1과의 결합이 감소되는 것을

확인하였다. 이때 ROS의 역할은 Trx, GSTM1 발현을 직접적으로 조절하는데 관여하기보다는 이들 단백질들이 ASK1과의 결합을 저해시키는 방식으로 세포사멸을 유도하는데 주된 작용을 하였다. 한편, Smad4 단백질은 TGFβ 신호전달체계에서 신호전달에 참여하는데 여기서는 TGF-β에 의한 종양 progression에 관여하는 표적유전자의 전사 활성화를 매개하며, Trx 와 GSTM1 또한 그 표적 유전자들중에 속하였다.

무엇보다도 TGF-β 발현억제에 따른 세포사멸유도가 ASK1을 매개하여 일어나는지를 확인하기 위하여 세포내 ASK1 발현을 저해시키는 siASK1을 전처리하고 shhTGF-β1 또는 2를 발현하는 아데노바이러스를 감염시켰을 때 TGF-β 발현억제에 따른 세포사멸이 현저히 감소된 것을 관찰하였다.

따라서 본 연구에서는 shhTGF-β1 또는 2를 발현하는 아데노바이러스에 의한 세포사멸은 ASK1의 활성화를 통해 p38 와 JNK의 활성화가 유도되면서 주로 기인된 것임을 알게 되었으며, 또한 ASK1의 활성화는 Trx 와 GSTM1 유전자의 발현감소와 Trx와 GSTM1이 각각 ASK1-Trx, ASK1-GSTM1에서 떨어져서 협동적으로 활성화됨을 알 수 있었다.

핵심되는 말 : TGF-β1, TGF-β2, 활성산소,ASK1, 종양살상 아데노바이러스, 세포사멸

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