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Distinct roles of transforming growth factor-β signaling and transforming growth factor-β receptor inhibitor SB431542 in the regulation of p21 expression

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ABSTRACT

Transforming growth factor-β (TGF-β) has both tumor suppressive and oncogenic activities. Autocrine TGF-β signaling supports tumor survival and growth in certain types of cancer, and the TGF-β signaling pathway is a potential therapeutic target for these types of cancer. TGF-β induces p21 expression, and p21 is considered as an oncogene as well as a tumor suppressor, due to its anti-apoptotic activity. Thus, we hypothesized that autocrine TGF-β signaling maintains the expression of p21 at levels that can support cell growth. To verify this hypothesis, we sought to examine p21 expression and cell growth in various cancer cells following the inhibition of autocrine TGF-β signaling using siRNAs targeting TGF-β signaling components and SB431542, a TGF-β receptor inhibitor. Results from the present study show that p21 expression and cell growth were reduced by knockdown of TGF-β signaling components using siRNA in MDA-MB231 and A549 cells. Cell growth was also reduced in p21 siRNA-transfected cells. Downregulation of p21 expression induced cellular senescence in MDA-MB231 cells but did not induce apoptosis in both cells. These data suggest that autocrine TGF-β signaling is required to sustain p21 levels for positive regulation of cell cycle. On the other hand, treatment with SB431542 up-regulated p21 expression while inhibiting cell growth. The TGF-β signaling pathway was not associated with the SB431542-mediated induction of p21 expression. Specificity protein 1 (Sp1) was downregulated by treatment with SB431542, and p21 expression was increased by Sp1 knockdown. These findings suggest that downregulation of Sp1 expression is responsible for SB43154-induced p21 expression.

1. Introduction

Transforming growth factor-β (TGF-β) is a secreted growth and differentiation factor that binds to TGF-β receptor II and recruits and phosphorylates TGF-β receptor I (Bassing et al., 1994; Wrana et al., 1994). The activated TGF-β receptor I exerts its biological functions through phosphorylation of intracellular Smad2 and Smad3, with subsequent interaction with Smad4 protein to form an oligomeric complex (Derynck et al., 1998; Massague, 1998). The Smad2/Smad4 and Smad3/Smad4 complexes can act as transcriptional activators or repressors in the nuclei (Massague and Chen, 2000). Due to its potent growth inhibitory activity in many cell types (Lyons and Moses, 1990), TGF-β has been considered as a tumor suppressor. Some cancer cells often lose their growth inhibitory responses to TGF-β through mutation of TGF-β receptors or Smads (Eppert et al., 1996; Hahn et al., 1996; Howe et al., 1998; Markowitz et al., 1995; Myeroff et al., 1995). These mutations have been suggested to promote tumor progression during the late stages of carcinogenesis (Wilentz et al., 2000). On the other hand, several studies have shown that mutations of TGF-β receptor II are rare in breast, endometrial, pancreatic, and lung carcinomas (Abe et al., 1996; Myeroff et al., 1995; Tomita et al., 1999). Similarly, analysis of Smad genes revealed no mutations, except Smad2 and Smad4, in tumors from the colon, breast, lung, and pancreas (Riggins et al., 1997). In addition, the Smad4 mutation is uncommon in breast and ovarian carcinomas (Schutte et al., 1996). These reports strongly suggest that TGF-β signaling is required for the progression of certain tumors.

TGF-β inhibits cell proliferation by inhibiting avian myelocytomatosis virus oncogene cellular homolog (c-Myc) expression and inducing p15 and p21 expression, which consequently causes retinoblastoma protein (pRB) hyper-phosphorylation (Datto et al., 1995; Li et al., 1995; Massague, 2000). Because of the inactivation of tumor repressor genes, such as pRB, and the overexpression of proto-oncogenes, such as c-Myc, carcinoma cells are often resistant to TGF-β-mediated growth inhibition while TGF-β signaling

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pathways remain functional. For example, DU145 human prostate adenocarcinoma cells are resistant to growth inhibition by TGF-β due to the mutation of pRB but are sensitive to target gene regulation by TGF-β (Murphy et al., 1991). Similarly, the failure of TGF-β-mediated inhibition of c-Myc expression causes resistance to its growth inhibition in MCF-10A human mammary epithelial cells and MDA-MB231 human breast adenocarcinoma cells, without causing abrogation of TGF-β signaling (Chen et al., 2001). Moreover, autocrine TGF-β signaling supports the survival and growth of MDA-MB231 cells while avoiding the growth inhibitory activity of autocrine TGF-β (Dumont et al., 2003; Lei et al., 2002). These studies indicate that autocrine TGF-β signaling is a potential therapeutic target for cancers that are resistant to growth inhibition by TGF-β.

p21 (also called WAF1, CAP20, cyclin-dependent kinase (CDK)-interacting protein 1 (Cip1), and Sdi1) is a member of the Cip/kinase inhibitory protein family of CDK inhibitors, which also includes p27 (Toyoshima and Hunter, 1994) and p57 (Lee et al., 1995), and can bind and inhibit a broad range of cyclin/CDK complexes (Harper et al., 1995). It plays an essential role in growth arrest after DNA damage (Brugarolas et al., 1995), and its overexpression leads to G1 and G2 or S-phase arrest (Niculescu et al., 1998; Ogryzko et al., 1997). Therefore, p21 was initially considered as a tumor suppressor (Franklin et al., 2000; Martin-Caballero et al., 2001; Poole et al., 2004; Topley et al., 1999). However, it was later suggested that p21 also acts as an oncogene, due to its pro-cancer and anti-apoptotic activities in some cancers (Gartel, 2006).

These reports led us to hypothesize that autocrine TGF-β signaling maintains the expression of p21 at levels that can support cell growth. To verify this hypothesis, we sought to examine p21 expression and cell growth in various cancer cells following the inhibition of autocrine TGF-β signaling using siRNAs targeting TGF-β signaling components and SB431542, a potent inhibitor of TGF-β receptor I (Inman et al., 2002).

2. Materials and methods

2.1. Reagents

SB431542, Cycloheximide, SP600125, and MG132 were purchased from Sigma-Aldrich (St. Louis, MO). TGF-β and TGF-β receptor I-specific polyclonal antibody (AF3025) were purchased from R&D Systems (Minneapolis, MN). Antibodies recognizing p15 (#4822), p16 (#4824), p18 (#2896), p21 (#2947), p27 (#3698), p57 (#2557), Smad2 (#3122), phosphorylated (phospho)-Smad2 (#3108), cleaved caspase-3 (#9664), caspase-3 (#9665), cleaved caspase-9 (#7237), caspase-9 (#9508), Akt (#4691), phosphor-Akt (#4060), specificity protein 1 (Sp1; #5931), phospho-JNK (#4668), JNK (#9258), and Bmi1 (#6964) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (MAB374)- and β1 integrin (MAB2252)-specific monoclonal antibodies were purchased from Millipore (Billerica, MA). Non-targeting siRNA (#D-001210-01) and siRNAs targeting TGF-β receptor I (#M-003929-02), Smad2 (#M-003561-01), p21 (#D-003471-01), ALK4 (transforming growth factor-β superfamily type I activin receptor-like kinase 4) (#M-004925-01), ALK7 (#M-004929-02), β1 integrin (#M-004506-00), Bmi1 (#M-005230-00), and specificity protein 1 (Sp1; #D-026959-00) were purchased from GE Healthcare (Seoul, Korea)

2.2. Expression plasmids

Human testis complementary DNA (Marathon cDNA, Clontech, Palo Alto, CA) was used as a template to amplify the full-length cDNA for p21 (Accession no. NM_000389). The following oligonucleotide primers were used for PCR with Advantage 2 polymerase (Clontech, Palo Alto, CA): 5′-GAACTCGAGGAGGCGCCATGTCAGAACCG-3′ (XhoI site underlined) and 5′-CCGGGATCCTCAGGGCTTCCTCTTGGAGAAGA-3′ (BamHI site underlined). The 500-bp PCR product was digested with XhoI and BamHI, after which it was cloned into the pcDNA3.1/myc-His(-) B vector (Invitrogen) that was digested with XhoI and BamHI.

2.3. Cell culture and transfection

MDA-MB231 human breast adenocarcinoma cells (ATCC no. HTB-26), MCF-7 human breast adenocarcinoma cells (ATCC no. HTB-22), MDA-MB435 human melanoma cells (ATCC no. HTB-129), A549 human lung carcinoma cells (ATCC no. CCL-185), HT-1080 human fibrosarcoma cells (ATCC no. CCL-121), HCT116 human colorectal carcinoma cells (ATCC no. CCL-247), SW-480 human colorectal carcinoma cells (ATCC no. CCL-228), Caco-2 human colorectal carcinoma cells (ATCC no. HTB-37), and COS-1 cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS). Transfections with siRNAs and plasmids were performed using Lipofectamine 2000, according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

2.4. Western blotting

Cells were lysed in buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, and the protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN]) for 10 min at 4 °C and then centrifuged. The soluble portion of the lysate was used for western blotting, which was performed by separating reduced samples on SDS-polyacrylamide gel electrophoresis, followed by electroblotting to polyvinylidene difluoride membranes. Enhanced chemiluminescence (GE Healthcare) was used to detect the bound antibody. The signal intensity of relevant bands from the western blot was quantitated using ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). Five micrograms of mRNA were used to synthesize first-strand cDNA, using the Superscript III First-Strand kit (Invitrogen). One microliter of cDNA was used as the template for PCR. Primer sets were as follows: TGF-β receptor I, 5′-GAGATTGTTGGTACCCAAG-3′ (forward) and 5′-TCAACTGATGGGTCAGAAGG-3′ (reverse); Smad2, 5′-CATCTTGCCATTCACGCCGC-3′ (forward) and 5′-CTGTAAAGGCCTGTTGTATC-3′ (reverse); p21, 5′-ACTTCGACTTTGTCACCGAG-3′ (forward) and 5′-ATTAGGGCTTCCTCTTGGAG-3′ (reverse); Sp1, 5′-CTGCCCTCCAATCCATTTTC-3′ (forward) and 5′-TTT GACCAGAACCATCCTGC-3′ (reverse); ALK4, 5′-CCATTTTCAATCTGGATGGG-3′ (forward) and 5′-CATCTCACATGAGGGATCTT-3′ (reverse); ALK7, 5′-GAAGTGTGTATGTCTTTTGT-3′ (forward) and 5′-TTGCAATTGTCCTTTGAACC-3′ (reverse); and GAPDH, 5′-GAAGCTCACTGGCATGGCCTT-3′ (forward) and 5′-CTCTCTTGCTCAGTGTCCTTGCT-3′ (reverse).

2.6. Cell proliferation assay

To compare the proliferation of SB431542- or TGF-β-treated cells with the proliferation of control cells, MDA-MB231 cells (5×10^3) were cultured in 96-well culture plates for 24 h, after which they were incubated in 1% FBS-supplemented medium with or without SB431542 (2 μM) or various concentrations of TGF-β for 48 h. To compare the proliferation of siRNA-transfected cells with the proliferation of non-targeting siRNA-transfected cells,

transfected cells were plated in 96-well culture plates and incubated in 5% FBS-supplemented medium for 48 h. Cell numbers were assessed using the MTT assay (Amresco, Solon, OH). MTT was added to each well, and then the plate was incubated at 37 °C. After 2 h of incubation, the absorbance was measured at 540 nm.

2.7. Senescence-associated β-galactosidase staining

Senescence-associated β-galactosidase staining was performed using the Senescence β-Galactosidase Staining Kit (#9860) according to the manufacturer's instructions (Cell Signaling

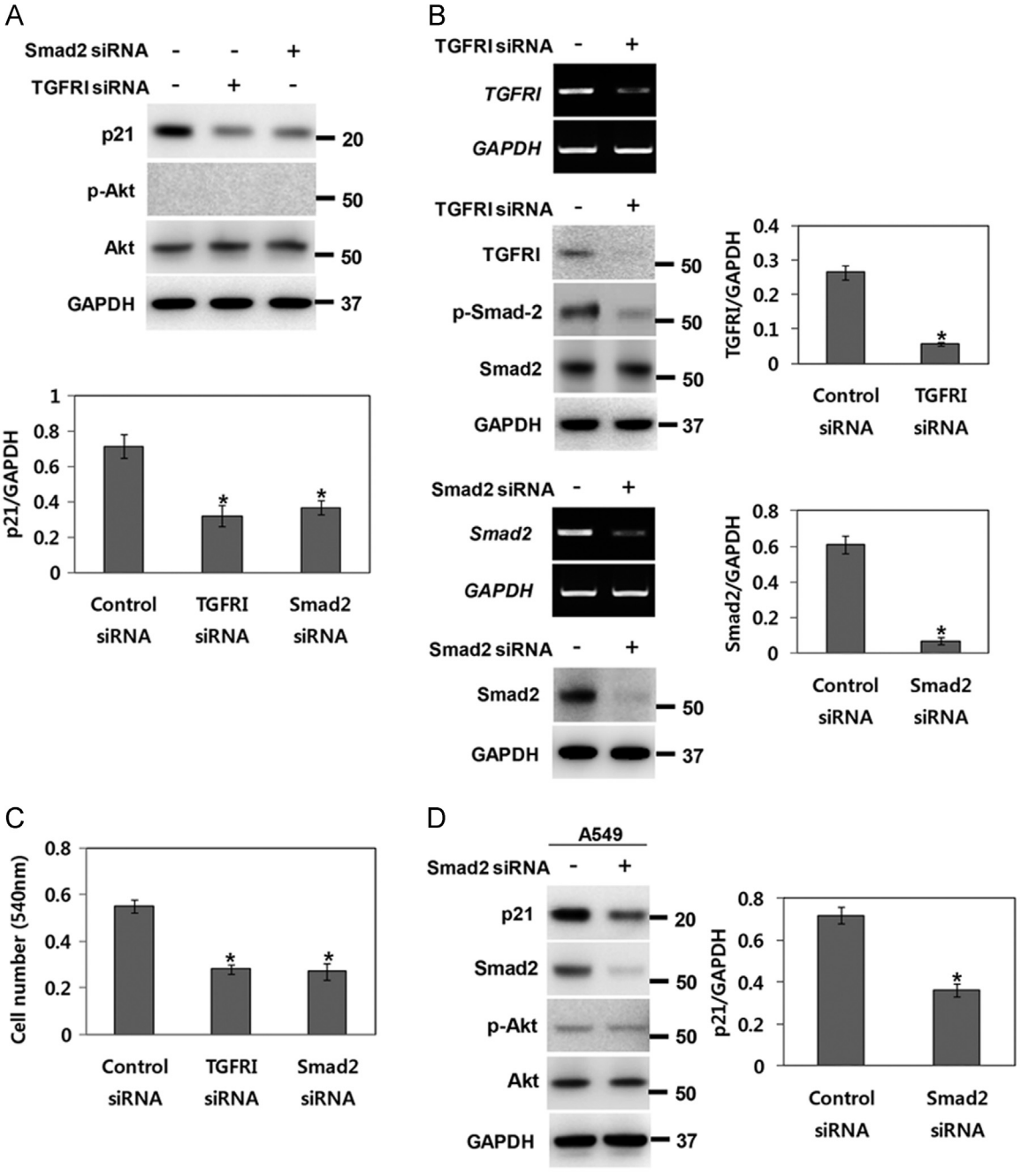

Fig. 1. Knockdown of TGF-β signaling components reduces p21 expression and cell proliferation. A. Expression of p21 in control siRNA-, TGF-β receptor I (TGFRI) siRNA-, or Smad2 siRNA-transfected MDA-MB231 cells. Cells were transfected with the indicated siRNA (100 nM) and cultured in 12-well plates at 37 °C. After 3 days of incubation, cells were serum starved in serum-free medium for 18 h and cell lysates were analyzed by western blotting using antibodies recognizing p21, phospho-Akt, Akt, or GAPDH. The bar graph shows the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus control siRNA. B. RT-PCR and western blot analysis were performed to show knockdown of TGFRI and Smad2. Cell lysates from the transfected cells were analyzed by western blotting using antibodies recognizing TGFRI, Smad2, or GAPDH. After TGFRI siRNA-transfected cells were treated with TGF-β (5 ng/ml) in serum-free medium for 1 h, cell lysates were also analyzed by western blotting with antibodies recognizing phospho-Smad2 or Smad2. The bar graph shows the ratio of TGFRI or Smad2 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus control siRNA. C. Effect of siRNA transfection on cell proliferation was evaluated in MDA-MB231 cells as described in Section 2. Transfection was performed using the indicated siRNAs (100 nM). Cell numbers were assessed by measuring the optical density at 540 nm. Data represent the mean and standard deviation of six experiments. $^{*}P < 0.05$ versus control siRNA. D. Expression of p21 in control siRNA- or Smad2 siRNA-transfected A549 cells. Cells were transfected with the indicated siRNA (100 nM) and cultured in 12-well plates for 3 days. After serum starvation for 18 h, cell lysates were analyzed by western blotting using antibodies recognizing p21, Smad2, phospho-Akt, Akt, or GAPDH. The bar graph shows the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus control siRNA.

Technology Inc.). After MDA-MB231 cells were transfected with siRNAs and cultured in 12-well culture plates at 37 °C for 7 days, the transfected cells were fixed using 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 min at room temperature, and rinsed in PBS. Then, the fixed cells were stained for senescence-associated β-galactosidase activity. Cells were washed and embedded in PBS, viewed in an inverted light microscope (IX51; Olympus) and documented by a digital imaging system (ProgRes CapturePro Version 2.8.8; Jenoptik).

2.8. Statistical analysis

Data are represented as means and standard deviations. Statistical analysis was performed using the unpaired Student's *t* test. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Knockdown of TGF-β signaling components reduces p21 expression and cell proliferation

We tested p21 expression after knocking down TGF-β receptor I and Smad2 in cells. MDA-MB231 cells that were transfected with siRNA against TGF-β receptor I or Smad2 exhibited decreases in p21 expression (Fig. 1A). Knockdown of TGF-β receptor I led to a significant decrease in Smad2 phosphorylation (Fig. 1B, upper panel). The efficient knockdown of TGF-β receptor I and Smad2 was confirmed by RT-PCR and western blot analyses (Fig. 1B). These data suggest that TGF-β signaling can contribute to the maintenance of p21 expression in these cells. Next, we examined whether blockade of autocrine TGF-β signaling by siRNA transfection reduces cell growth. The MTT assay data showed that cell proliferation was inhibited by knockdown of TGF-β signaling components (Fig. 1C). It has been reported that TGF-β induces Akt activation and enhances cell proliferation (Kattla et al., 2008; Kim et al., 2002). Results from western blotting of cell lysates show no activated Akt in MDA-MB231 cells, suggesting that cell growth inhibition by knockdown of TGF-β signaling components is independent of Akt signaling (Fig. 1A). The effect of Smad2 knockdown on p21 expression was further examined in other cell types. Knockdown of Smad2 downregulated p21 expression in A549 cells (Fig. 1D), but not in MDA-MB435, COS-1, MCF-7, and HT-1080 cells (Fig. S1). Phosphorylation of Akt was not affected by Smad2 siRNA in all cells examined (Figs. 1D and S1). These data indicate that knockdown of TGF-β signaling components leads to the downregulation of p21 expression in a cell-type specific manner.

3.2. Downregulation of p21 expression reduces cell proliferation

Because it was suggested that p21 acts as an oncogene by inhibiting apoptosis (Gartel, 2006), we examined the influence of

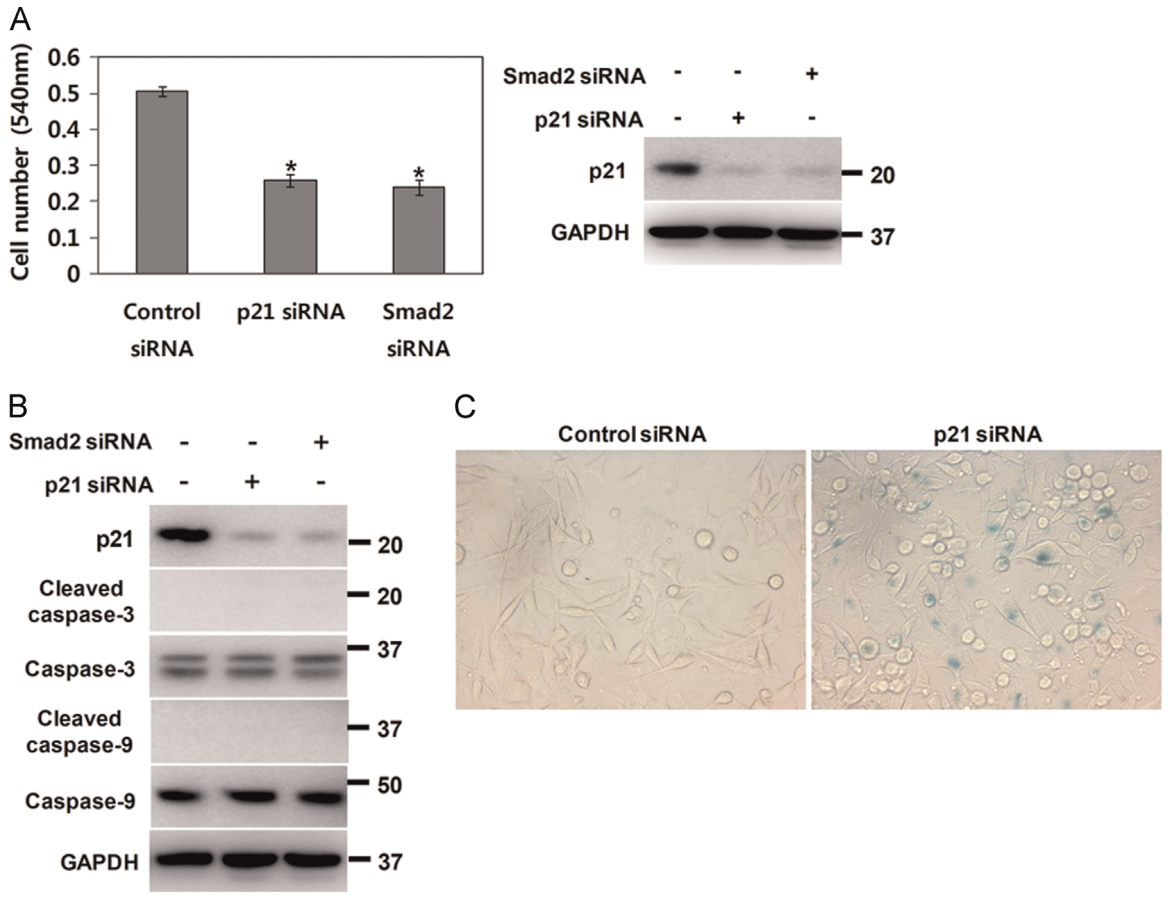

Fig. 2. Downregulation of p21 expression results in the reduced cell proliferation and induction of cellular senescence. A. Effect of siRNA transfection on cell proliferation was evaluated in MDA-MB231 cells as described in Section 2. Transfection was performed using the indicated siRNAs (100 nM). Western blot analysis was performed to show p21 expression. Cell numbers were assessed by measuring the optical density at 540 nm. Data represent the mean and standard deviation of six experiments. $^{*}P < 0.05$ versus control siRNA. B. Effect of siRNA transfection on apoptosis in MDA-MB231 cells. Cells were transfected with the indicated siRNA (100 nM) and cultured in 12-well plates at 37 °C. After 7 days of incubation, cell lysates were analyzed by western blotting using antibodies recognizing p21, cleaved caspase-3, caspase-3, cleaved caspase-9, caspase-9, or GAPDH. C. Influence of p21 knockdown on cellular senescence was evaluated in MDA-MB231 cells as described in Section 2.

p21 knockdown on proliferation of MDA-MB231 and A549 cells. Expression of p21 was suppressed substantially by p21 siRNA (Figs. 2A and 2SA, right panel). When the cell number was measured by the MTT assay, the results showed that cell proliferation was significantly decreased by p21 knockdown (Figs. 2A and S2A, left panel). A similar reduction in cell proliferation and down-regulation of p21 expression was also observed in Smad2 siRNA-transfected cells (Figs. 2A and S2A). Next, we examined whether apoptosis is induced by knockdown of p21 or Smad2. When p21 or Smad2 was knocked down in these cells and cell lysates were analyzed by western blotting with an anti-cleaved caspase-3 antibody or an anti-cleaved caspase-9 antibody, the results revealed that apoptosis was not observed in siRNA-transfected cells (Figs. 2B and S2B). A senescence-like phenotype was seen in MDA-MB231 cells but not in A549 cells after p21 knockdown (Figs. S2C and S3). Thus, we examined whether the morphological change in

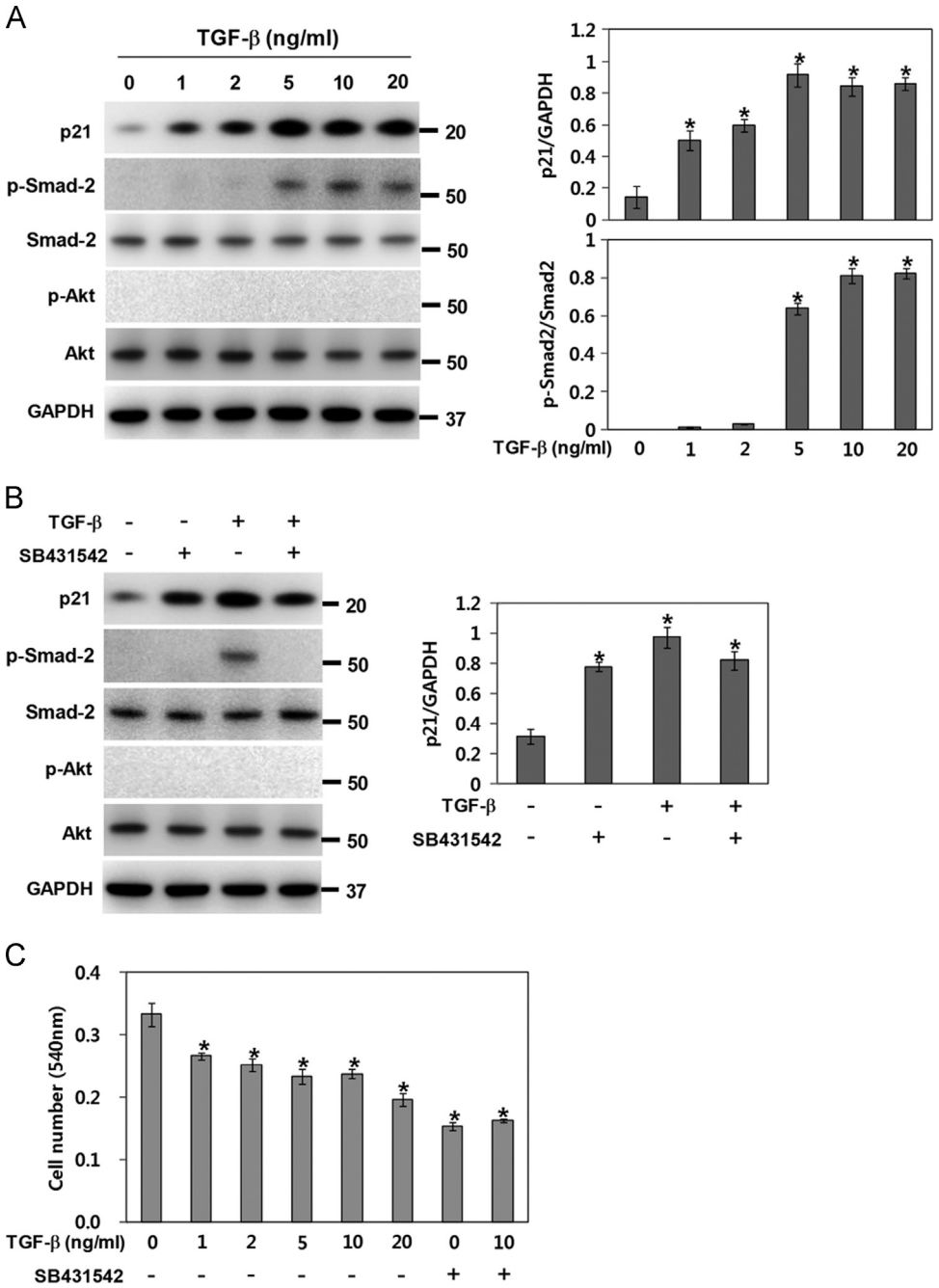

Fig. 3. SB431542 inhibits the growth of MDA-MB231 cells but induces p21 expression. A. Concentration-dependent p21 expression in TGF-β-treated MDA-MB231 cells. After serum-starved cells were treated with different concentrations of TGF-β (0, 1, 2, 5, 10, and 20 ng/ml) for 8 h, cell lysates were analyzed by western blotting using antibodies specific to p21, phospho-Smad2, Smad2, phospho-Akt, Akt, or GAPDH. The bar graphs show the ratio of p21 to GAPDH or phospho-Smad2 to Smad2. Data represent the mean and standard deviation of three experiments. $^*P < 0.05$ versus untreated control. B. Effect of SB431542 on p21 expression in MDA-MB231 cells. After serum-starved cells were incubated in the presence and absence of SB431542 (2 μM) and/or TGF-β (5 ng/ml) for 8 h, cell lysates were analyzed by western blotting using antibodies specific to p21, phospho-Smad2, Smad2, phospho-Akt, Akt, or GAPDH. The bar graph shows the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. $^*P < 0.05$ versus untreated control. C. Effect of SB431542 and TGF-β on the proliferation of MDA-MB231 cells was evaluated as described in Section 2. Cells were cultured in 96-well plates at 37 °C for 24 h and then incubated in the presence and absence of SB431542 (2 μM) and/or TGF-β (0, 1, 2, 5, 10, and 20 ng/ml) for 48 h. Cell numbers were assessed using the MTT assay. Data represent the mean and standard deviation of six experiments. $^*P < 0.05$ versus untreated control.

MDA-MB231 cells was due to the cellular senescence. When senescence-associated β-galactosidase staining was performed, where only cells in senescence developed blue staining, the results showed that β-galactosidase activity was greatly increased by p21 knockdown (Fig. 2C). Taken together, these experimental data suggest that autocrine TGF-β signaling maintains the expression of p21 at levels that can support cell growth.

3.3. SB431542 treatment inhibits the growth of MDA-MB231 cells but induces p21 expression

First, to determine the effective concentration of TGF-β to induce p21 expression, cells were treated with various concentrations of TGF-β, and cell lysates were analyzed by western blotting with an anti-p21 antibody. TGF-β induced p21 expression in a

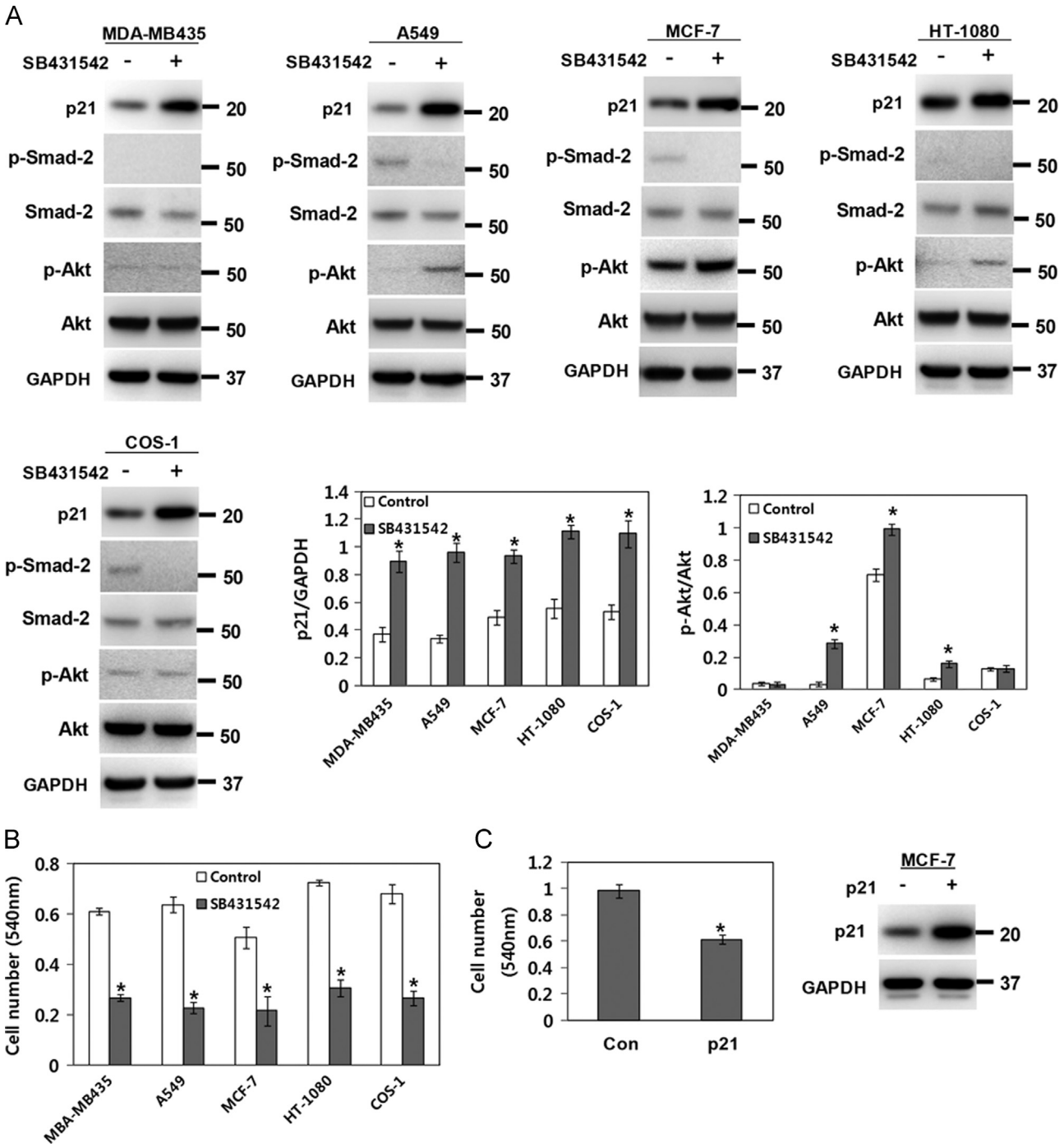

Fig. 4. SB431542 induces p21 expression in various cell types. A. Effect of SB431542 on p21 expression and phosphorylation of Smad2 and Akt in MDA-MB435, A549, MCF-7, HT-1080, and COS-1 cells. After cells were treated with 2 μM SB431542 for 8 h, cell lysates were analyzed by western blotting using antibodies specific to p21, phospho-Smad2, Smad2, phospho-Akt, Akt, or GAPDH. The bar graphs show the ratio of p21 to GAPDH or phospho-Akt to Akt. Data represent the mean and standard deviation of three experiments. $^*P < 0.05$ versus untreated control. B. Effect of SB431542 on the proliferation of MDA-MB435, A549, MCF-7, HT-1080, and COS-1. Cells were cultured in 96-well plates at 37 °C for 24 h and then incubated in the presence and absence of SB431542 (2 μM) for 48 h. Cell numbers were assessed using the MTT assay. Data represent the mean and standard deviation of six experiments. $^*P < 0.05$ versus untreated control. C. Effect of p21 overexpression on the proliferation of MCF-7 cells. After cells were transfected with p21 expression vector for 48 h and transfected cells were further cultured in 96-well plates at 37 °C for 48 h, cell numbers were assessed using the MTT assay. Western blot analysis was performed to show p21 expression. Data represent the mean and standard deviation of six experiments. $^*P < 0.05$ versus control vector.

concentration-dependent manner up to 5 ng/ml (Fig. 3A). However, Akt was not activated by TGF-β treatment in MDA-MB231 cells (Fig. 3A). Then, we examined the expression of p21 in MDA-MB231 cells that were treated with SB431542 and/or TGF-β. Unlike previous reports (Halder et al., 2005; Soto-Cerrato et al., 2007), p21 expression was up-regulated in SB431542-treated cells (Fig. 3B). TGF-β also induced p21 expression and Smad2 phosphorylation. Although treatment with SB431542 had a little effect on TGF-β-induced p21 expression, it completely abolished Smad2 phosphorylation (Fig. 3B). Akt was not affected by SB431542 treatment (Fig. 3B). Next, the influence of TGF-β and SB431542 on the growth of MDA-MB231 cells was examined. Cells were treated with TGF-β or SB431542, and cell proliferation was measured by the MTT assay. Treatment with TGF-β inhibited cell growth in a concentration-dependent manner (Fig. 3C). This inhibition was also observed in SB431542-treated cells, regardless of TGF-β treatment (Fig. 3C). Because various CDK inhibitors are involved in cell growth arrest, we examined the expression of other CDK inhibitors in SB431542-treated cells. Results from western blotting show that SB431542 slightly down-regulated p18 expression and did not alter p27 or p57 expression, thus suggesting that its effect on p21 expression was specific (Fig. S4). Expression of p15 and p16 was not detected in these cells (Fig. S4). Next, the effect of SB431542 on p21 expression was examined in other cell types. Although SB431542 efficiently inhibited Smad2 phosphorylation, it induced p21 expression in MDA-MB435, A549, MCF-7, HT-1080, and COS-1 cells (Fig. 4A). These data indicate that SB431542 treatment elicits the induction of p21 expression in various cell types. Unexpectedly, Akt activation was increased by SB431542 treatment in A549, MCF-7, and HT-1080 cells (Fig. 4A). Inhibition of cell proliferation was observed in these cells after SB431542 treatment (Fig. 4B). Moreover, consistent with the previous reports (Niculescu et al., 1998; Ogryzko et al., 1997), cell growth was greatly reduced by overexpression of p21 using expression vector in MCF-7 cells (Fig. 4C).

3.4. SB431542-mediated induction of p21 expression is independent of the TGF-β signaling pathway

Because SB431542 is a potent inhibitor of TGF-β receptor I (Inman et al., 2002) and an inducer of p21 expression in various cell types, we further examined the involvement of TGF-β signaling in this process. In HCT116 colorectal carcinoma cells, which are defective in TGF-β receptor II (Markowitz et al., 1995), treatment with SB431542 up-regulated p21 expression, whereas treatment with TGF-β had no effect (Fig. 5A). To examine whether Smad

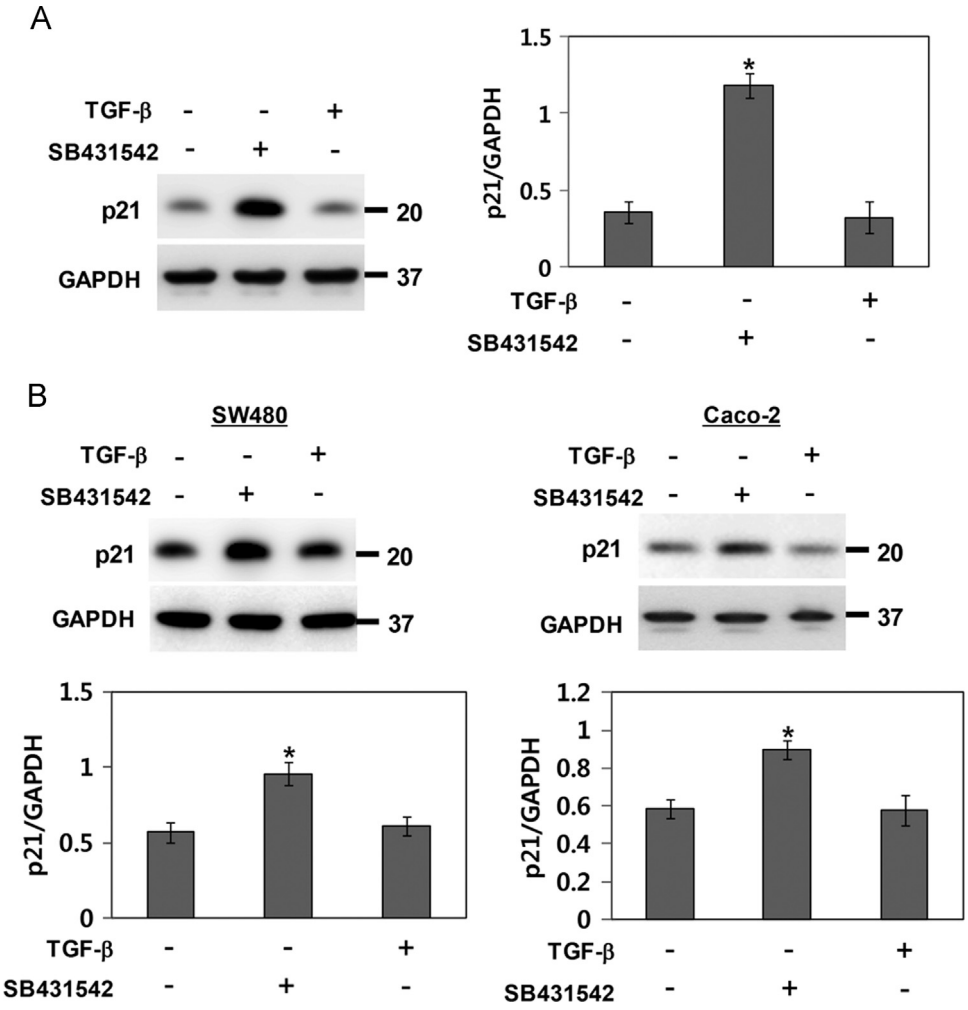

Fig. 5. TGF-β signaling pathway is not involved in the SB431542-mediated induction of p21 expression. A. Effect of SB431542 and TGF-β on p21 expression in HCT116 cells. After cells were incubated in the presence and absence of SB431542 (2 μM) or TGF-β (10 ng/ml) for 8 h, cell lysates were analyzed by western blotting using p21- and GAPDH-specific antibodies. The bar graph shows the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus untreated control. B. Effect of SB431542 and TGF-β on p21 expression in SW480 and Caco-2 cells. After cells were incubated in the presence and absence of SB431542 (2 μM) and/or TGF-β (10 ng/ml) for 8 h, cell lysates were analyzed by western blotting using p21- and GAPDH-specific antibodies. The bar graph shows the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus untreated control.

signaling is involved in the regulation of p21 expression by SB431542, Smad4-deficient SW480 and Caco-2 colon cancer cells were treated with SB431542 and TGF-β, and p21 expression was assessed by western blot analysis. As shown in Fig. 5B, SB431542 induced p21 expression in both cells, whereas p21 expression was not altered in TGF-β-treated cells. This indicates that SB431542 induces p21 expression in a Smad-independent manner. SB431542 has been reported to inhibit ALK4 and ALK7, which are very highly related to TGF-β receptor I in their kinase domains (Inman et al., 2002). Therefore, the cellular expression of p21 was assessed in MDA-MB231 cells that were transfected with siRNA against ALK4 or ALK7. Western blot analysis of cell lysates revealed that p21 expression was not altered in ALK4 siRNA- or ALK7 siRNA-transfected cells (Fig. S5). Overall, these data suggest that the TGF-β signaling pathway is not involved in the SB431542-mediated induction of p21 expression.

3.5. SB431542 does not affect p21 protein stability

The suppression of proteasomal degradation stabilizes and increases levels of p21 protein (Bloom et al., 2003). Thus, we performed a Cycloheximide chase experiment with p21 vector-transfected COS-1 cells to examine whether p21 induction occurs through the SB431542-mediated stabilization of p21 protein. Transfected cells were treated with SB431542 or MG-132 (positive control) in the presence of Cycloheximide, which inhibits new protein synthesis (Schneider-Poetsch et al., 2010). The decrease in p21 expression was not affected by SB431542 treatment, whereas a reduction in the decrease in p21 expression was observed in MG132-treated cells (Fig. 6A). This suggests that SB431542 does not influence p21 protein stability. Next, we determined whether SB431542 induces p21 expression at the transcriptional level. Treatment with SB43154 gradually increased p21 mRNA (Fig. 6B). These findings suggest that SB431542 induces p21 expression through transcriptional regulation, but not through the stabilization of p21 protein.

3.6. SB431542-induced p21 expression is not dependent on the functions of p53, JNK, Bmi1, and β1 integrin

Several mechanisms have been proposed to control p21 transcription, including p53, JNK, Bmi1 and β1 integrin. Therefore, we

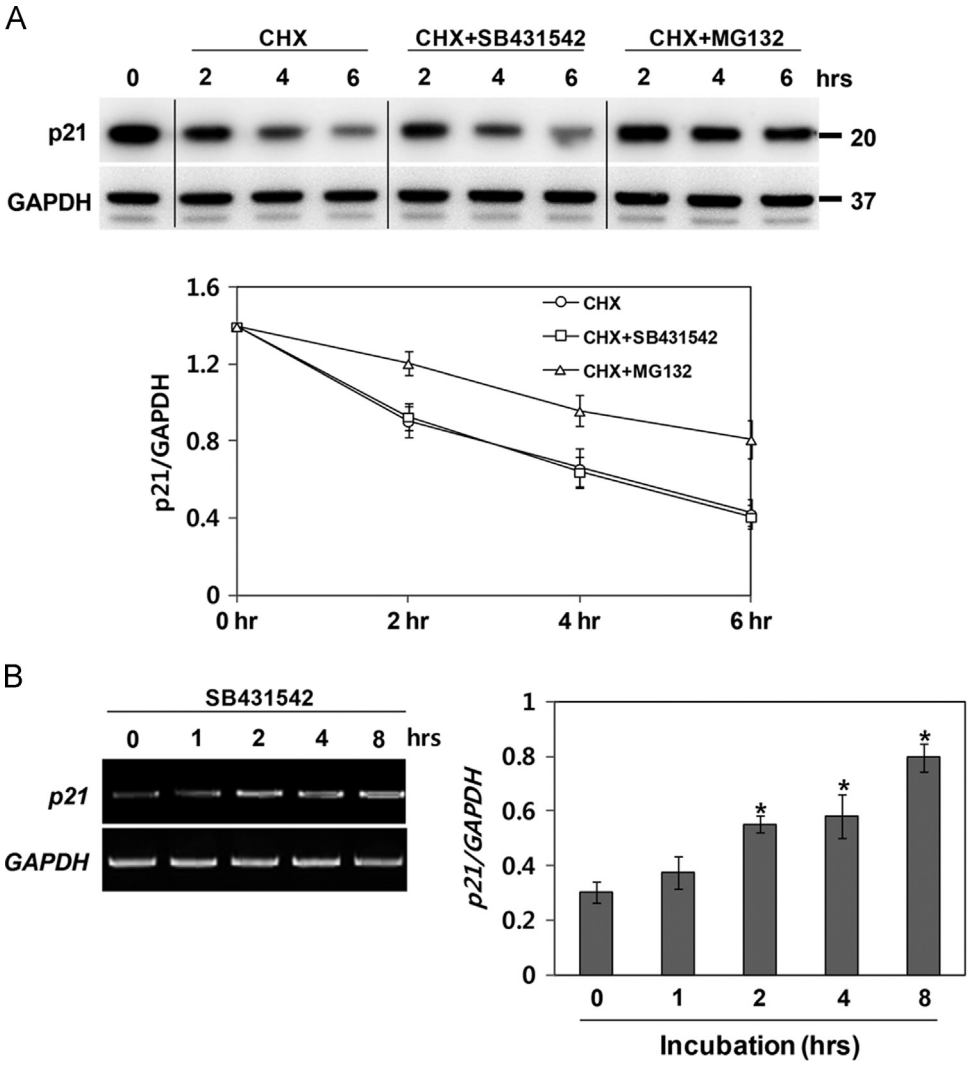

Fig. 6. SB431542 does not affect p21 protein stability. A. Cycloheximide (CHX) chase experiment. COS-1 cells were transfected with p21 expression vector for 48 h. Transfected cells were then treated with 2 μM SB431542 or 7 μM MG-132 in the presence of 100 μg/ml CHX for the indicated time points, and cell lysates were analyzed by western blotting using p21- and GAPDH-specific antibodies. The line graphs show the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. B. Analysis of p21 mRNA in SB431542-treated HCT116 cells. After cells were treated with 2 μM SB431542 for the indicated time points, semi-quantitative RT-PCR (20 cycles) was performed. The bar graph shows the ratio of p21 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus untreated control.

investigated the roles of these signaling factors in the SB431542-mediated induction of p21 expression. HCT116 cells, which lack p53 and have defective TGF-β signaling (Bunz et al., 1998), were used. SB431542 treatment induced p21 expression (Fig. 5A), thus ruling out the involvement of p53 in the induction process. SP600125, which inhibits JNK signaling, has been shown to up-regulate p53 expression and its transcriptional target, p21, in HepG2 cells and HCT116 $p53^{+/+}$ cells (Kuntzen et al., 2005). Thus, we tested whether SB431542-induced p21 expression is affected upon SP600125 treatment in HCT116 $p53^{-/-}$ cells. SB431542-induced p21 protein expression was not altered in the presence of SP600125 (Fig. S6A). As expected, treatment with SP600125 alone did not induce p21 expression in these cells (Fig. S6A). JNK phosphorylation was greatly reduced by treatment with SP600125 but not with SB431542 (Fig. S6A). These results indicate that the JNK signaling pathway is not involved in SB431542-induced p21 expression. In addition, Bmi1 is a member of the polycomb family that has been shown to repress p21 expression in neural stem cells and granule cell progenitors by directly binding to its promoter (Fasano et al., 2007; Subkhankulova et al., 2010). We examined whether Bmi1 plays a role in SB431542-induced p21 expression in HCT116 cells by transfecting cells with Bmi1 siRNA and treating them with or without SB431542. Expression of p21 was not affected by Bmi1 knockdown in the presence and absence of SB431542 (Fig. S6B), which rules out the involvement of Bmi1 in this induction process. Furthermore, the increased expression of β1 integrin has been shown to up-regulate p21 transcription through a p53-independent pathway (Fang et al., 2007). Thus, we investigated the role of β1 integrin in the SB431542-mediated induction of p21 expression. Western blotting results clearly showed a rapid increase in β1 integrin expression in SB431542-treated cells (Fig. S6C). However, β1 integrin siRNA-transfected cells did not exhibit changes in p21 expression in the presence and absence of SB431542 (Fig. S6D), thereby excluding the involvement of β1 integrin in SB431542-induced p21 expression. Collectively, these experimental data suggest that p53, JNK, Bmi1, and β1

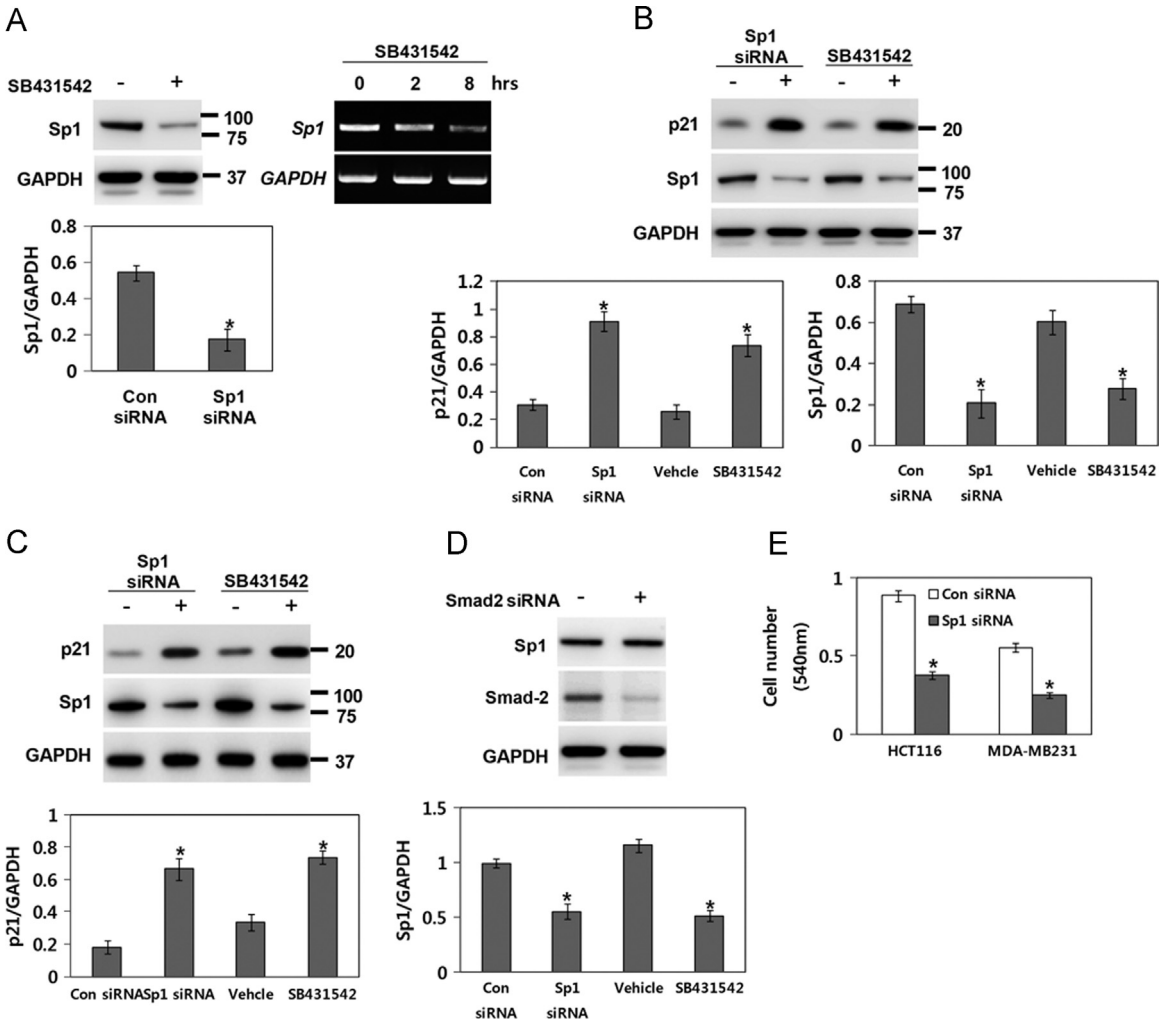

Fig. 7. SB431542-induced p21 expression is mediated by Sp1 downregulation. A. Influence of SB431542 on Sp1 expression in HCT116 cells. After cells were treated with 2 μM SB431542 for 8 h, cell lysates were analyzed by western blotting using Sp1- and GAPDH-specific antibodies. RT-PCR was also performed to show mRNA expression of Sp1 or GAPDH (right panel) after treating cells with 2 μM SB431542 for 2 h and 8 h. The bar graph shows the ratio of Sp1 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus untreated control. B and C. Expression of p21 in Sp1 siRNA-transfected HCT116 (B) and MDA-MB231 cells (C). Cells were transfected with control or Sp1 siRNA (100 nM) and cultured in 12-well plates at 37 °C. After 3 days of incubation, cell lysates were analyzed by western blotting using antibodies recognizing p21, Sp1, or GAPDH. Expression levels of p21 and Sp1 were also analyzed after treating cells with 2 μM SB431542 for 8 h. The bar graphs show the ratio of p21 to GAPDH and the ratio of Sp1 to GAPDH. Data represent the mean and standard deviation of three experiments. $^{*}P < 0.05$ versus untreated control. D. Effect of Smad2 knockdown on Sp1 expression. Cells were transfected with control or Smad2 siRNA (100 nM) and cultured in 12-well plates at 37 °C. After 3 days of incubation, cell lysates were analyzed by western blotting using antibodies recognizing Sp1, Smad2, or GAPDH. E. Effect of siRNA transfection on cell proliferation was evaluated in HCT116 and MDA-MB231 cells as described in Section 2. Transfection was performed using the indicated siRNAs (100 nM). Data represent the mean and standard deviation of six experiments. $^{*}P < 0.05$ versus control siRNA.

integrin are not involved in the SB431542-mediated induction of p21 expression.

3.7. Downregulation of Sp1 expression contributes to SB431542-induced p21 expression

The transcription factor Sp1 has been shown to regulate p21 transcription (Li and Davie, 2010). Thus, a role for Sp1 in SB431542-mediated p21 induction was investigated. A significant decrease in Sp1 protein expression was observed in HCT116 and MDA-MB231 cells that were treated with SB431542 (Fig. 7A and C). RT-PCR analysis also showed that levels of Sp1 mRNA were decreased by treatment with SB431542 (Fig. 7A, right panel), indicating that the mechanism of this control occurs at a transcriptional level. Next, we tested whether p21 expression was affected by downregulation of Sp1 expression. Western blotting showed that knockdown of Sp1 by siRNA induced p21 expression in both cells (Fig. 7B and C). Downregulation of Sp1 protein was confirmed in Sp1 siRNA-transfected cells (Fig. 7B and C). Similar expression levels of Sp1 and p21 were also observed in SB431542-treated cells (Fig. 7B and C). Next, we examined whether inhibition of Smad signaling could lead to the downregulation of Sp1 expression. When Smad2 was knocked down in MDA-MB231 cells and cell lysates were analyzed by western blotting with an anti-Sp1 antibody, the results showed no alteration of Sp1 expression (Fig. 7D). We further investigated whether downregulation of Sp1 expression by siRNA transfection reduces cell proliferation. The MTT assay data showed that cell proliferation was reduced by knockdown of Sp1 in HCT116 and MDA-MB231 cells (Fig. 7E). Overall, these experimental data suggest that SB431542-mediated down-regulation of Sp1 leads to reduced cell proliferation at least in part through p21 induction.

4. Discussion

The present study shows that cell growth was reduced in exogenous TGF-β-treated MDA-MB231 breast cancer cells. Blockade of autocrine TGF-β signaling by siRNA transfection also reduced cell growth. These findings contradict a report showing that the failure to inhibit c-Myc expression by TGF-β causes the resistance to its growth inhibition in MDA-MB231 cells (Chen et al., 2001). Because of the opposing roles of TGF-β signaling in cell growth, we postulate that the underlining mechanisms for cell growth inhibition are different in these two circumstances. TGF-β has been shown to mediate cell cycle arrest by inducing the expression of p15 (Li et al., 1995) and/or p21 (Datto et al., 1995). In the present study, we also showed that p21 expression was induced in TGF-β-treated cells, along with the concomitant growth inhibition of MDA-MB231 cells. Moreover, cell growth was greatly reduced by overexpression of p21 using expression vector. When autocrine TGF-β signaling was inhibited by siRNA transfection, p21 expression and cell proliferation decreased. Cell growth was also reduced by knockdown of p21 in MDA-MB231 cells. The expression of p21 has been shown to inhibit apoptosis in cancer, resulting in cell survival and growth (Gartel, 2006; Martin-Caballero et al., 2001). It was suggested that p21 acts as a positive regulator of the cell cycle due to its role of an assembly factor for cyclinD-cdk4/cdk6 (LaBaer et al., 1997). In fact, it was shown that Notch-mediated repression of p21 expression reduces cyclinD-cdk4 complex formation and nuclear targeting with subsequent cell cycle arrest (Gartel and Radhakrishnan, 2005; Noseda et al., 2004). Cell cycle arrest can lead to apoptosis or cellular senescence (Blagosklonny, 2011; Pucci et al., 2000). Consistent with the function of p21 as a positive regulator of the cell cycle, our experimental data also show that downregulation of p21 expression induces cell growth inhibition and cellular senescence. There are also some reports that autocrine TGF-β signaling supports the survival and growth of MDA-MB231 cells (Dumont et al., 2003; Lei et al., 2002). Thus, along with the data for the reduced cell proliferation in siRNA-transfected cells, these results suggest that autocrine TGF-β signaling mediates the survival and growth of MDA-MB231 cells by maintaining basal p21 expression and consequently preventing cell cycle arrest. Our findings also suggest that cell growth can be suppressed in TGF-β-responsive cancers by both exogenous TGF-β treatment and the inhibition of autocrine TGF-β signaling, which are mediated by different underlying mechanisms.

It is likely that SB431542 mediates the inhibition of MDA-MB231 cell proliferation by affecting autocrine TGF-β signaling and non-TGF-β signaling pathway. The blockade of autocrine TGF-β signaling by siRNA transfection resulted in a decrease in p21 expression and the concomitant reduction in cell proliferation, whereas SB431542 treatment elicited p21 overexpression and simultaneously inhibited cell proliferation. The induction of p21 expression by SB431542 was unexpected, as SB431542 has been shown to potently inhibit TGF-β receptor I and block TGF-β-induced p21 promoter activation and protein expression (Halder et al., 2005; Inman et al., 2002). The discrepancies in results from the present study compared with other studies may be due to the fact that different cell lines were used. Previous reports showed that SB431542 also inhibits the activity of ALK4 and ALK7 without affecting the ERK, JNK, or p38 MAPK pathway (Inman et al., 2002; Laping et al., 2002). However, the activity of ALK4 and ALK7 is unlikely to be implicated in SB431542-induced p21 expression, as knockdown of ALK4 and ALK7 did not affect p21 expression.

The transcription factor Sp1 plays an important role in regulating the expression of genes that are involved in many cellular processes. It functions by binding to the promoter regions of its target genes (Li and Davie, 2010). The control of Sp1 expression occurs at the transcriptional, translational, or post-translational level. A decrease in Sp1 mRNA and protein by SB431542 treatment indicates that Sp1 expression is affected at a transcriptional level. There are several examples to regulate Sp1 gene expression. The Sp1 promoter is positively regulated by Sp1 itself, nuclear factor Y, E2F transcription factor, and estrogen-related receptor α1 (Nicolas et al., 2003, 2001; Sumi and Ignarro, 2005). Cell cycle regulatory proteins including CDK4, SKP2, Rad51, BRCA2, E2F-DP1, cyclin D1, Stat3, and Rb also activate the Sp1 promoter, whereas p53 and NF-κB inhibit its activation (Tapias et al., 2008). Even though there is no report showing the direct correlation between SB431542 and these regulators, we presume that there is a possibility for their relationship. However, because the Sp1 protein positively regulates its own promoter activity (Nicolas et al., 2001), and a decrease in Sp1 protein through post-translational regulation leads to reduced Sp1 transcription, we cannot exclude the possibility that Sp1 is downregulated at the post-translational level by SB431542 treatment.

Sp1 silencing induces p21 and p27 expression to inhibit cell proliferation, clonogenicity, and the anchorage-independent growth of nasopharyngeal carcinoma cells (Zhang et al., 2014). It also activates the transcription of Bmi1 and centromere protein H in nasopharyngeal carcinoma (Wang et al., 2013; Zhao et al., 2012). Bmi1 promotes tumor progression by inhibiting the transcription of tumor suppressors, such as p53 (Calao et al., 2013), p21 (Fasano et al., 2007), INK4a, and p19 (Bruggeman et al., 2007). Therefore, we assumed that the SB431542-mediated down-regulation of Sp1 expression could reduce the transcription of its downstream oncogenes, thus leading to the up-regulation of CDK inhibitors, including p21. However, we did not observe alterations in p21 expression by Bmi1 knockdown, which suggests that Bmi1 is unlikely to be implicated in SB431542-induced p21 expression.

At present, it remains unclear how Sp1 expression is downregulated by SB431542 treatment and its downregulation elicits

p21 induction. Thus, further studies are needed to elucidate the underlying mechanisms of SB431542-mediated downregulation of Sp1 expression and p21 induction by Sp1 downregulation, which will lead to apply SB431542 to both TGF-β-unresponsive and TGF-β-responsive cancer cells.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2015.07.032.

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