



Downregulation of the DNA repair enzyme apurinic/apyrimidinic endonuclease 1 stimulates transforming growth factor- β 1 production and promotes actin rearrangement



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ABSTRACT

The DNA repair enzyme apurinic/apyrimidinic endonuclease 1 (APE1) plays a central role in base excision repair and functions as a reductive activator of various transcription factors. Multiple other functionalities have been ascribed to APE1 in addition to these major functions. A recent study showed that APE1 knockdown upregulated the expression of a set of genes related to extracellular matrix (ECM) production, indicating an additional novel biological role for this enzyme. Based on this finding, we have investigated the effect of APE1 downregulation on ECM-related gene expression and its biological consequences. Endogenous APE1 expression was downregulated in human cervical carcinoma HeLa cells and human lung carcinoma A549 cells using siRNA. When the expression of six ECM-related genes (*TGFB1*, *LAMC1*, *FN1*, *COL1A1*, *COL3A1*, and *COL4A1*) was evaluated, we found that APE1 knockdown upregulated the expression of *TGFB1* in both cell lines. APE1 downregulation promoted actin rearrangement, inducing F-actin accumulation in HeLa cells and the dissipation of stress fibers in A549 cells. We also discovered that APE1 knockdown enhanced cellular motility in A549 cells, which was suppressed by the inhibition of transforming growth factor (TGF)- β 1 signaling. These results suggested that APE1 controls the organization of actin cytoskeleton through the regulation of TGF- β 1 expression, providing novel insights into the biological significance of APE1.

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1. Introduction

Apurinic/apyrimidinic endonuclease 1 (APE1) is a homolog of *Escherichia coli* exonuclease III and primarily functions as a DNA repair enzyme [1]. APE1 has an apurinic/apyrimidinic

endonuclease domain at its C-terminal region that is highly conserved in various organisms. In addition, mammalian APE1 has a nuclear localization signal and a redox domain in its N-terminal domain. APE1 is indispensable for cellular and organismal survival, as demonstrated by early embryonic lethality in APE1 knockout mice; attempts to isolate stable APE1-knockout cells have also been unsuccessful [2,3]. Mammalian APE1 is also involved in the activation of transcription factors. The DNA binding affinities of various transcription factors, such as activator protein 1 (AP-1), nuclear factor- κ B (NF- κ B), and hypoxia-inducible factor-1 α (HIF-1 α), are modulated by the redox status of the cysteine residues in their DNA binding domains [4–6]. The redox activity of APE1 causes the reduction of redox-sensitive cysteine residues, leading to their activation. In addition to these major functions, various studies have reported the other functions of APE1, such as redox-independent transcriptional activation [7–9], RNA quality control [10,11], and telomere maintenance [12]. Therefore, APE1 has a wide range of functions that potentially influence many biological processes.

Abbreviations: AP-1, activator protein 1; APE1, apurinic/apyrimidinic endonuclease 1; B2M, β -2-microglobulin; *COL1A1*, collagen type I α 1; *COL3A1*, collagen type III α 1; *COL4A1*, collagen type IV α 1; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; *FN1*, fibronectin 1; HIF-1 α , hypoxia-inducible factor-1 α ; *LAMC1*, laminin γ 1; NF- κ B, nuclear factor- κ B; PBS, phosphate buffered saline; PMS, phenazine methosulfate; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species; TGF, transforming growth factor; *TGFB1*, TGF- β 1; TRITC, tetramethylrhodamine isothiocyanate; WST-1, water-soluble tetrazolium salts.

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However, the multifunctional properties of APE1 complicate the elucidation of specific functions regulating cellular physiology and increase the difficulty of determining the corresponding mechanism. In addition, the inability to establish an APE1-deficient cell line or APE1 knockout mouse has hampered investigations into this protein. Vascotto et al. recently performed a study analyzing the effect of APE1 downregulation on global gene expression by microarrays using a conditional knockout system [13]. The authors reported significant changes in the expression of genes related to multiple cellular functions including lipid metabolism, cell cycle regulation, protein synthesis, and DNA repair. Notably, a set of genes related to the production of the extracellular matrix (ECM) was upregulated as a result of APE1 downregulation. The aforementioned APE1 functions, including cell cycle regulation and DNA repair, are well characterized and closely associated with the enzymatic activity of the protein. However, the involvement of APE1 in ECM production was unexpected. We were intrigued by this finding; therefore, we sought to decipher the mechanism by which APE1 regulates ECM-related gene expression, and determine the biological consequences of this novel aspect of APE1 function.

2. Materials and methods

2.1. Reagents

Lipofectamine[®] 2000, Stealth RNAi[™] siRNA Negative Control LO GC (Control siRNA), and Stealth RNAi[™] siRNA targeting APE1 (ID: HSS100557 and HSS100556, hereafter referred to as siAPE1 #1 and siAPE1 #2, respectively) were purchased from Life Technologies (Carlsbad, CA, USA). Anti-APE1 antibody, anti-actin antibody, and horseradish peroxidase-conjugated anti-mouse and anti-goat antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-transforming growth factor (TGF)- β 1 antibody (clone 9016) was obtained from R&D Systems (Minneapolis, MN, USA). TGF- β type I receptor kinase inhibitor LY364947 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin was obtained from Sigma–Aldrich (St. Louis, MO, USA). Water-soluble tetrazolium salts (WST-1) and 1-methoxy phenazine methosulfate (PMS) were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

2.2. Cell culture and siRNA transfection

Human cervical carcinoma HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂. Human lung carcinoma A549 cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS at 37 °C in 5% CO₂. The cells were transfected with APE1-targeting siRNA or control siRNA using Lipofectamine 2000. Following siRNA transfection, the medium was replaced with fresh medium and the transfected cells were used for subsequent analyses.

2.3. Western blot analysis

Cells were lysed with lysis buffer (50 mM Tris–HCl [pH 7.5], 1% (v/v) Triton X-100, 5% (v/v) glycerol, 5 mM EDTA, 150 mM NaCl) and centrifuged at 20,000 \times g for 15 min at 4 °C. The supernatant was collected, and 3 \times Laemmli's sample buffer (0.1875 M Tris–HCl [pH 6.8], 15% (v/v) β -mercaptoethanol, 6% (w/v) SDS, 30% (v/v) glycerol and 0.006% (w/v) bromophenol blue) was added to each supernatant. Equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Advantec TOYO, Tokyo, Japan). The membranes were blocked with 10 mM Tris–HCl (pH

7.4), 0.1 M NaCl, and 0.1% (v/v) Tween-20 (TBST) supplemented with 5% (w/v) nonfat skim milk for 1 h. Subsequently, the membranes were probed with specific primary antibodies diluted with TBST supplemented with 5% (w/v) nonfat skim milk overnight at 4 °C. The membranes were then probed with horseradish peroxidase-conjugated secondary antibodies and the blots developed using a Western Lightning[™] Chemiluminescence Reagent Plus kit (Perkin Elmer Life Sciences, Boston, MA, USA).

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the Relia Prep[™] RNA Cell Mini-prep System (Promega Corporation, Madison, WI, USA), as per the manufacturer protocols. The RNA sample (2 μ g) was reverse-transcribed using the ReverTra Ace[®] qPCR RT Master Mix (TOYOBO, Osaka, Japan). The obtained cDNA was subjected to real-time PCR analysis using a LightCycler Nano System (Roche Applied Science, Mannheim, Germany), with the samples being prepared using FastStart Essential DNA Green Master mix (Roche Applied Science). The sequences of the PCR primers used in this study are as follows: TGF- β 1 (*TGFB1*), 5'-TGC TGA GGC TCA AGT TAA AA-3' and 5'-TCC GGT GAC ATC AAA AGA TA-3'; laminin γ 1 (*LAMC1*), 5'-CTG TTG TTC CCA AGA CAA AG-3' and 5'-TCT CCA AAG TAG CCA TCA TC-3'; fibronectin 1 (*FN1*), 5'-ATT TCA TGT CAT CCT GTT GG-3' and 5'-CTT TCA GTG CCT CCA CTA TG-3'; collagen type I α 1 (*COL1A1*), 5'-ATG TTC AGC TTT GTG GAC CT-3' and 5'-GTG ATT GGT GGG ATG TCT T-3'; collagen type III α 1 (*COL3A1*), 5'-CTA CTT CTC GCT CTG CTT CA-3' and 5'-ACA TAT TTG GCA TGG TTC TG-3'; collagen type IV α 1 (*COL4A1*), 5'-ATG GAA TTG TGG AAT GTC AG-3' and 5'-AGG CAA CTC TCT CCT TTT TG-3'; and β -2-microglobulin (*B2M*), 5'-TTC TGG CCT GGA GGC TAT C-3' and 5'-TCA GGA AAT TTG ACT TTC CAT TC-3'. The following cycle conditions were applied for PCR analysis: initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. The relative mRNA level of each ECM-related gene was normalized to that of *B2M*, which was used as the internal control.

2.5. WST-1 assay

Cells (1000 per well) were seeded into each well of 96-well plates containing growth medium 24 h after siRNA transfection. The medium was replaced with medium supplemented with 1% FBS at 48 h post-transfection; subsequently, 10 μ L WST-1 solution (3.6 μ g/ μ L WST-1, 70 ng/ μ L 1-methoxy PMS in 20 mM HEPES-KOH [pH 7.4]) was added to each well 48, 72, and 96 h post-transfection. The cells were incubated for 2 h at 37 °C, and the absorbance of each well was recorded at 450 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. F-actin staining

Cells were seeded to collagen-coated glass coverslips 24 h after siRNA transfection. Following incubation for 24 h or 48 h, the cells were fixed with 3.7% (w/v) paraformaldehyde/phosphate buffered saline (PBS) for 15 min at room temperature, and subsequently permeabilized with 0.1% (v/v) Triton X-100/PBS for 5 min at room temperature. The cells were then incubated with 1% (v/v) TRITC-conjugated phalloidin/PBS for 30 min at room temperature in the dark. The coverslips were mounted with Prolong Gold antifade reagent (Life Technologies). Fluorescent microscopic analysis and image acquisition were performed using an Olympus BX61 microscope (Olympus, Tokyo, Japan) equipped with a CCD camera. F-actin focal accumulation was evaluated by measuring the

fluorescent intensities and area sizes of F-actin foci in over 200 randomly chosen cells per condition using the Image J software (National Institutes of Health, Bethesda, MD, USA). F-actin focal accumulation was quantified by multiplying the focal fluorescence intensity with its size. The average F-actin focal accumulation for each condition was determined by dividing the sum of F-actin focal accumulation by the cell number. Relative F-actin focal accumulation was calculated by normalizing the average F-actin accumulation for each condition to that of the control.

2.7. Wound healing assay

Cells (2.8×10^4 cells) were seeded onto each side of an Ibidi 2-well culture-insert (Ibidi, Planegg, Germany) 24 h after siRNA transfection, and incubated for 24 h. The culture-insert was removed to create an area containing no cells, and the medium replaced with RPMI containing 1% FBS. The gap area was photographed at $400\times$ magnification using an Olympus IX71 light microscope (Olympus, Tokyo, Japan) at 0 and 48 h after the

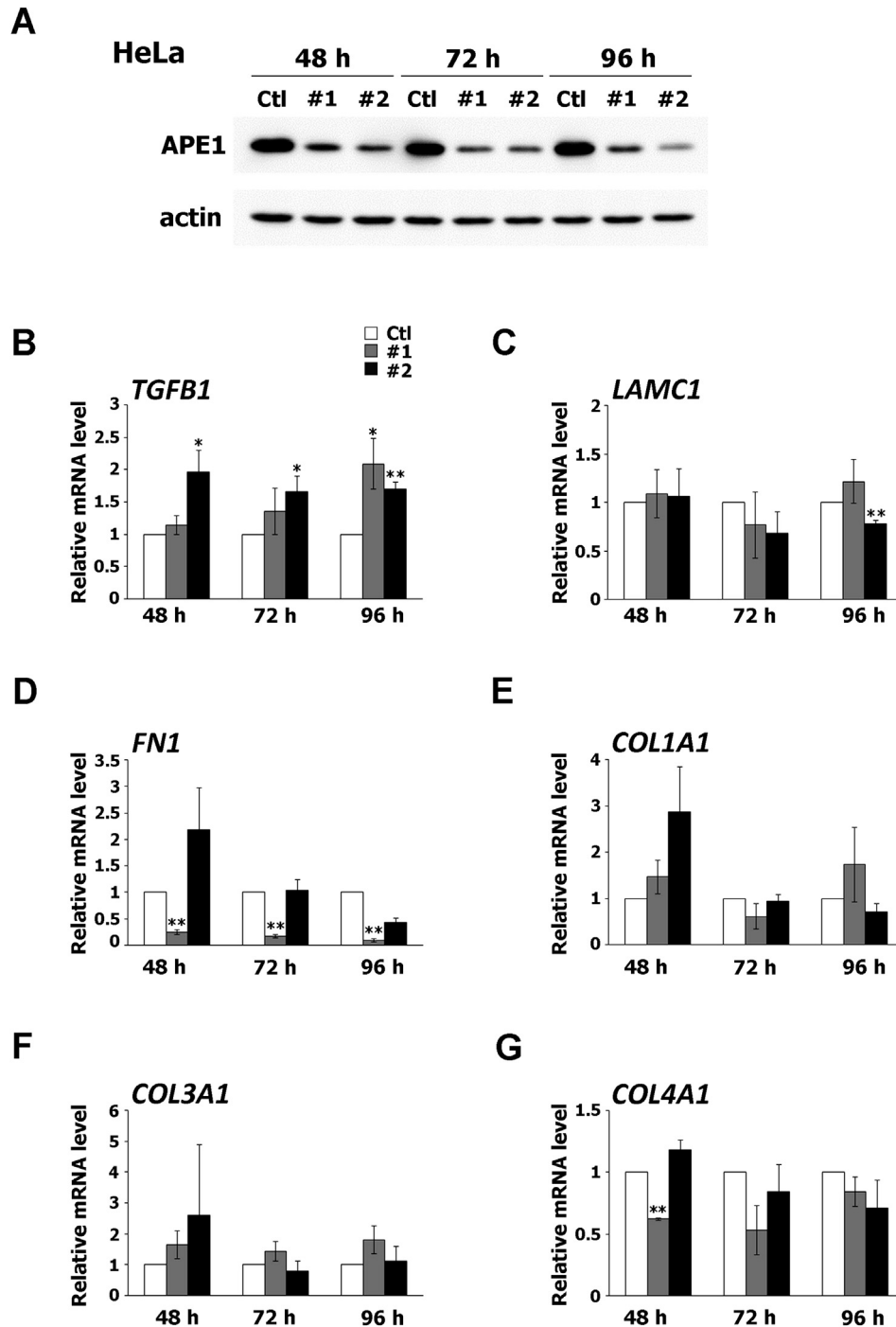


Fig. 1. Effect of APE1 downregulation on the expression of ECM-related genes in HeLa cells. (A) APE1 protein levels in HeLa cells transfected with control siRNA (Ctl), siAPE1 #1 (#1), or siAPE1 #2 (#2) and collected 48, 72, and 96 h after transfection were determined by western blot. Actin was used as loading control. Representative blots are shown. (B–G) Effect of APE1 downregulation on the expression of ECM-related genes. The mRNA levels in HeLa cells transfected with Ctl, #1, or #2 and collected 48, 72, and 96 h after transfection were analyzed by qRT-PCR. The mRNA levels were expressed as the ratio relative to the control group. (B) *TGFβ1* (C) *LAMC1* (D) *FN1* (E) *COL1A1* (F) *COL3A1* (G) *COL4A1*. All data are presented as mean \pm S.D. of three independent experiments. *p < 0.05; **p < 0.01 vs. Ctl (Student's *t*-test).

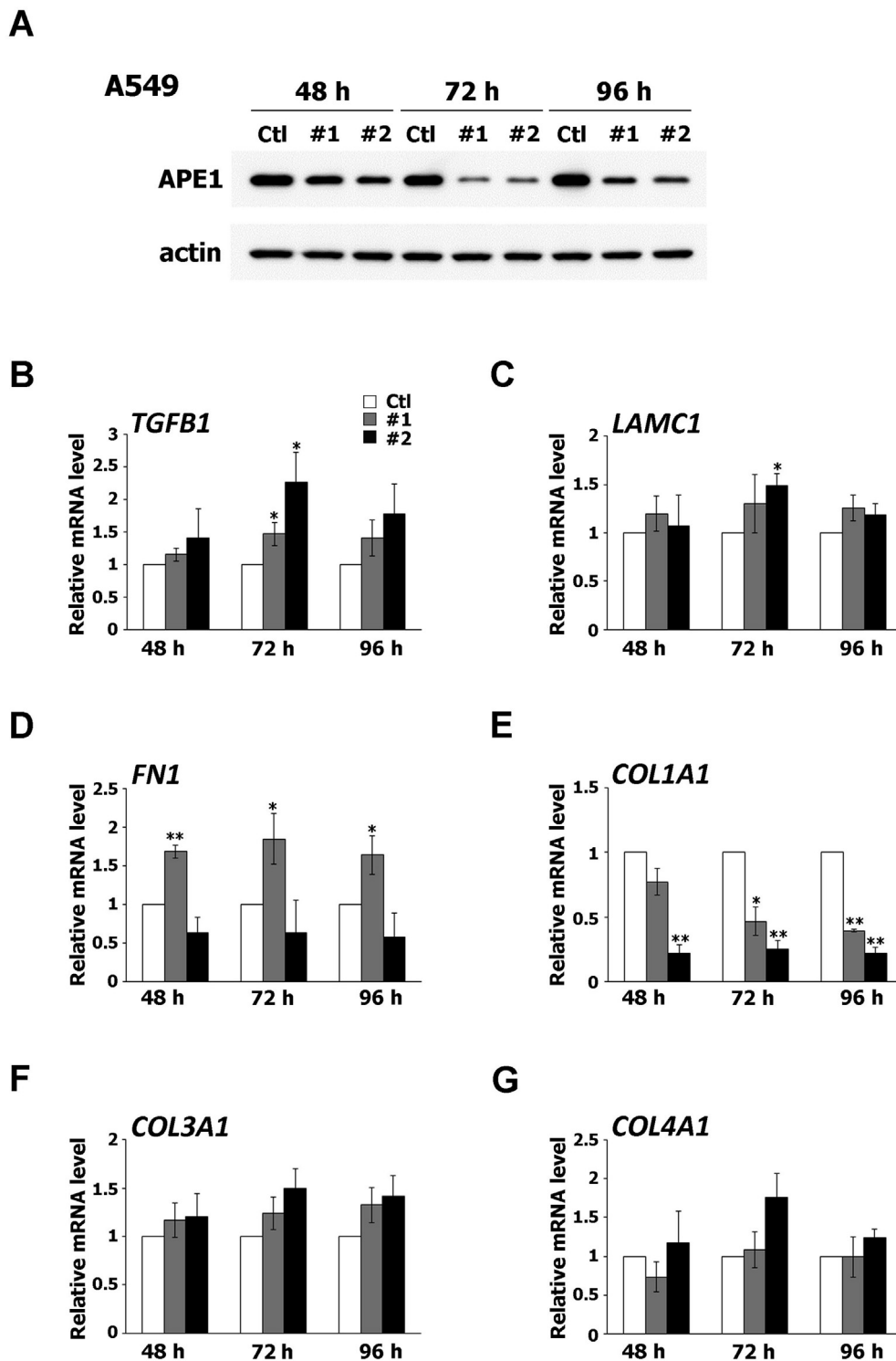


Fig. 2. Effect of APE1 downregulation on the expression of ECM-related genes in A549 cells. (A) APE1 protein levels in A549 cells transfected with control siRNA (Ctl), siAPE1 #1 (#1), or siAPE1 #2 (#2) and collected 48, 72, and 96 h after transfection were determined by western blot. Actin was used as loading control. Representative blots are shown. (B–G) Effect of APE1 downregulation on the expression of ECM-related genes. The mRNA levels of A549 cells transfected with Ctl, #1, or #2 and collected 48, 72, and 96 h after transfection were analyzed by qRT-PCR. The mRNA levels were expressed as the ratio relative to the control group. (B) *TGFB1* (C) *LAMC1* (D) *FN1* (E) *COL1A1* (F) *COL3A1* (G) *COL4A1*. All data are presented as mean \pm S.D. of three independent experiments. * $p < 0.05$; ** $p < 0.01$ vs. Ctl (Student's *t*-test).

medium change. Cell migration was evaluated by counting the number of cells that migrated to the gap area.

2.8. Statistical analysis

All results are displayed as mean \pm standard deviation (S.D.) of at least three independent experiments. Statistical analyses were performed using the Student's *t*-test. *p*-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of APE1 downregulation on the expression of ECM-related genes

The effect of siRNA on APE1 protein expression was verified in human cervical carcinoma HeLa cells and human lung carcinoma A549 cells at 48, 72, and 96 h after the transfection of control siRNA or the two types of APE1-targeting siRNA (siAPE1 #1 and #2). There was a decrease in APE1 protein expression 48 h after siRNA transfection, as seen in Figs. 1A and 2A; this effect was sustained for up to 96 h in both cell lines. The microarray analysis performed in a previous study revealed that the conditional knockdown of APE1 modified the expression of several ECM-related genes [13]. This finding was re-evaluated in our experimental settings by qRT-PCR analysis in HeLa and A549 cells. Six ECM-related genes, *TGFB1*, *LAMC1*, *FN1*, *COL1A1*, *COL3A1*, and *COL4A1*, reported to be upregulated as a result of APE1 downregulation, were selected as analysis targets [13]. *TGFB1* expression in HeLa cells was increased by the introduction of both siAPE1 #1 and #2 (Fig. 1B). Expression of *LAMC1* was downregulated 96 h after siAPE1 #2 transfection (Fig. 1C). The introduction of siAPE1 #1 (and not siAPE1 #2) effected a significant decrease in *FN1* expression (Fig. 1D), whereas siAPE1 did not cause any significant changes in the expression of either *COL1A1* or *COL3A1* (Fig. 1E and F). *COL4A1* expression was downregulated 48 h after the introduction of siAPE1 #1 (Fig. 1G). The same analyses conducted with A549 cells revealed that the introduction of siAPE1 #1 and #2 led to an elevation in *TGFB1* expression, with a peak observed at 72 h post-transfection (Fig. 2B). On the other hand, the expression of other ECM-related genes did not increase significantly, with the exception of *FN1*, following the introduction of siAPE1 #1 (Fig. 2C–G). The level of *TGFB1* expression was similarly upregulated by APE1 siRNA in both cell lines, which suggested that APE1 regulates the gene expression of TGF- β 1.

3.2. Effect of APE1 downregulation on cellular proliferation

The effect of siRNA oligonucleotide introduction on cellular viability or proliferation was examined by subjecting the transfected HeLa and A549 cells to a WST-1 assay. While neither of the siRNA constructs caused cytotoxicity in HeLa cells, the cells transfected with siAPE1 #1 demonstrated reduced proliferation, compared to those transfected with control siRNA and siAPE1 #2 (Fig. 3A). This suppressive effect of siAPE1 #1 on cell proliferation was observed to increase in a time-dependent manner. Similarly, siAPE #1 (and not siAPE #2 or the control siRNA) was observed to attenuate the proliferation of A549 cells (Fig. 3B). These results implied that despite the near-equivalent efficacy of siAPE1 #1 and #2 on APE1 knockdown, the resulting biological effects were not identical. As the induction of TGF- β 1 was more potent in cells (of both cell lines) transfected with siAPE1 #2 than those transfected with siAPE1 #1 (Figs. 1B and 2B), this disparity in cell proliferation is unlikely to be related to TGF- β 1-dependent inhibition of cell growth [14], but rather more likely to be due to a non-specific side

effect of siAPE1 #1 [15]. Therefore, we used siAPE1 #2 but not #1 to avoid unwanted effects in the subsequent experiments.

3.3. Effect of APE1 downregulation on actin cytoskeleton organization

TGF- β has been associated with cytoskeletal rearrangement [16]; therefore, we attempted to determine if APE1 downregulation affected actin cytoskeleton organization via TGF- β 1. Following the suppression of APE1, its impact on F-actin organization was monitored in HeLa cells. APE1-downregulated cells displayed a strong accumulation of F-actin around the central region of the cell (Fig. 4A). When APE1-downregulated cells were treated with an anti-TGF- β 1 antibody or TGF- β receptor kinase inhibitor, LY364947, both treatments resulted in a significant reduction in F-actin focal accumulation (Fig. 4B), suggesting the involvement of TGF- β 1 in APE1 downregulation-induced actin rearrangement. The same analysis performed in A549 cells resulted in the dissipation of actin stress fibers, which were evident in control cells, as a result of APE1 downregulation (Fig. 4C), unlike the HeLa cells. Because of the difficulty involved in the quantification of the dissipation of stress fibers, an assay was required to examine the involvement of TGF- β 1 in the APE1 siRNA-induced actin rearrangement in A549 cells. TGF-

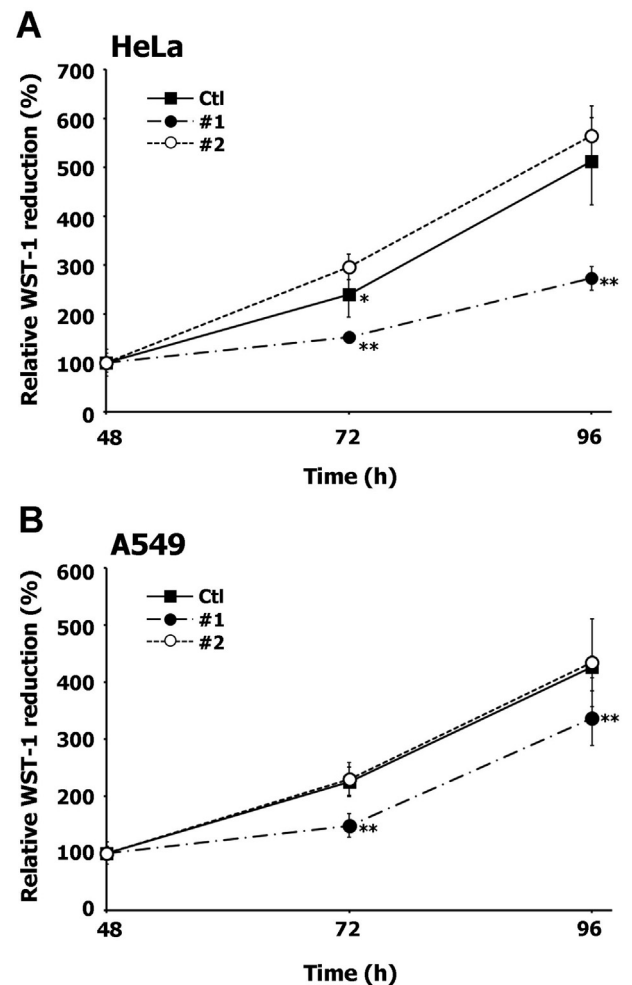


Fig. 3. Effect of APE1 downregulation on cellular viability/proliferation. WST-1 assay was performed 48, 72, and 96 h post-transfection in (A) HeLa cells and (B) A549 cells. The reduced WST-1 levels were expressed as percentages relative to the WST-1 levels at 48 h. All data are expressed as mean \pm S.D. of six independent experiments. **p* < 0.05; ***p* < 0.01 vs. Ctl (Student's *t*-test).

β is reported to stimulate cellular migration, which was accompanied by the reduction of stress fiber formation [17]. Therefore, the effect of APE1 downregulation on A549 cellular motility was tested using a wound healing assay. APE1 downregulation led to a significant increase in the number of migrated cells (Fig. 4D); the cell number was diminished by treatment with anti-TGF- β 1 antibody or LY364947. These results indicated that APE1 controls actin cytoskeleton organization, through the regulation of TGF- β 1 expression.

4. Discussion

The aim of this study was to elucidate the effect of APE1 downregulation on ECM-related gene expression and cellular function. APE1 expression was transiently inhibited using APE1-targeting siRNA in two cell lines, A549 cells and HeLa cells. In this study, qRT-PCR analysis showed that APE1 downregulation clearly increased *TGFB1* gene expression in both cell lines (Figs. 1B and 2B). This result suggested that APE1 regulates the expression of TGF- β 1. TGF- β is a multifunctional cytokine that modulates a variety of cellular functions, including cellular proliferation, and differentiation [18]. Although the precise transcriptional regulatory

mechanism controlling the expression of *TGFB1* remains unclear, it has been suggested that reactive oxygen species (ROS) are involved in the upregulation of *TGFB1* gene expression [19]. APE1 has been shown to influence intracellular ROS levels [20,21]; therefore, siRNA-induced downregulation of APE1 could have increased TGF- β 1 expression by enhancing ROS production. Regulation of microRNA expression by APE1 is another possible transcriptional regulatory mechanism of TGF- β 1. Dai et al. reported that APE1 downregulation affected the expression of various microRNAs, including miR-29b [22]. Because miR-29b is believed to repress TGF- β expression [23], it could be hypothesized that APE1 downregulation increases TGF- β gene expression via miR-29b regulation.

The expression of several ECM-related genes, such as *LAMC1*, *FN1*, *COL1A1*, *COL3A1*, and *COL4A1*, was also upregulated by APE1 knockdown, in parallel with the upregulated expression of *TGFB1* in a previous study [13]. The effect of APE1 siRNA on the expression of these five genes analyzed in this study revealed no significant changes (Figs. 1C–G and 2C–G). This inconsistency might be a result of the different methodologies used in the two studies (microarray vs. qPCR). Furthermore, analysis of the effect of APE1 siRNA on ECM-related gene expression in HeLa and A549 cells revealed the increase in TGF- β 1 to be the only consistent change in

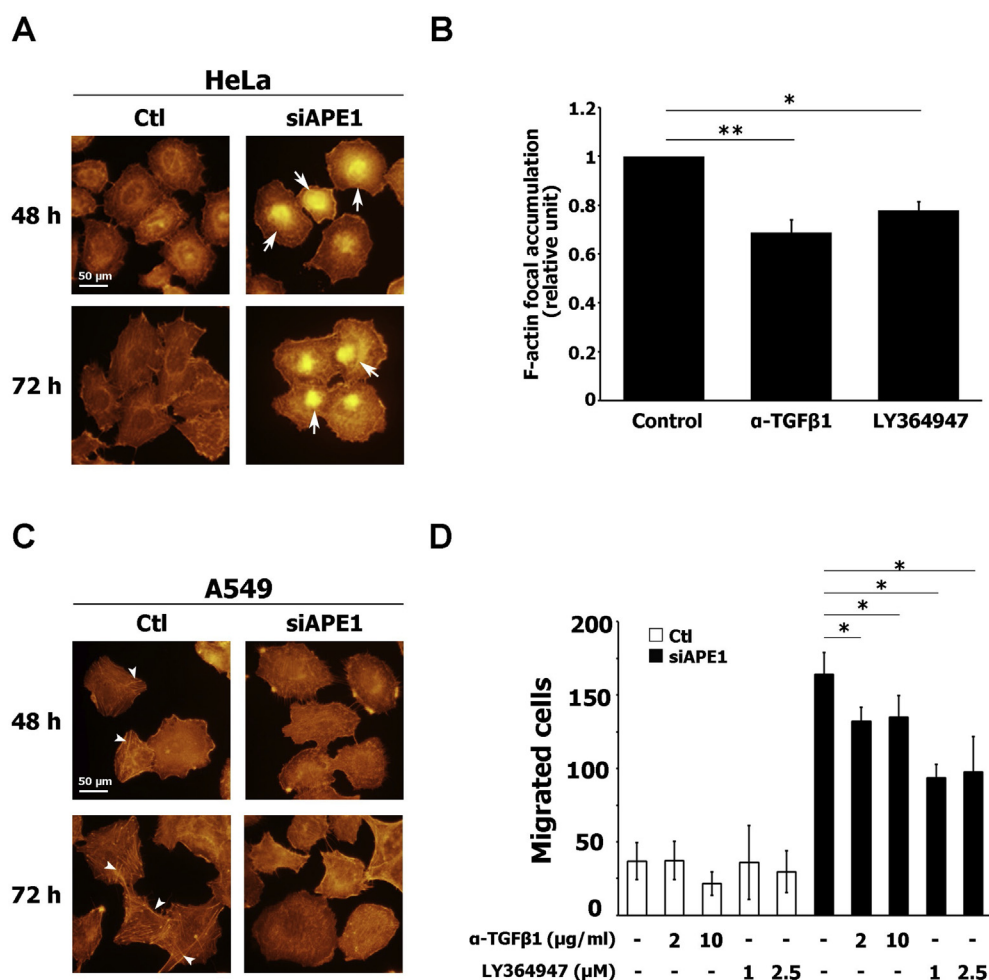


Fig. 4. Effect of APE1 downregulation on actin cytoskeleton organization. (A, C) F-actin was visualized by TRITC-conjugated phalloidin staining at 48 h and 72 h after transfection in (A) HeLa cells and (C) A549 cells. Arrows and arrowheads indicate F-actin focal accumulation and actin stress fibers, respectively. (B) Effect of TGF- β signaling inhibition on F-actin focal accumulation after APE1 downregulation. F-actin localization in HeLa cells, treated with the anti-TGF- β 1 antibody or LY364947 after transfection with siAPE1 #2, was visualized. F-actin focal accumulation was expressed as the ratio relative to the control. All data are expressed as mean \pm S.D. of three independent experiments. * p < 0.05; ** p < 0.01 (Student's t -test). (D) Effect of TGF- β signaling inhibition on cellular motility promoted by APE1 downregulation. The number of cells that migrated to the gap area was counted 48 h after the removal of inserts. All data are presented as mean \pm S.D. of three independent experiments. * p < 0.05 (Student's t -test).

both cell lines; the transcriptional response of the other genes differed, following APE1 knockdown (Figs. 1B–G and 2B–G). These results suggested that APE1 downregulation causes different effects, depending on the cell type. The cause of this phenomenon remains unclear. However, since APE1 downregulation stimulated TGF- β 1 expression under all experimental conditions, TGF- β 1 gene expression is highly likely to be regulated by APE1.

In this study, APE1 downregulation caused rearrangements in the actin cytoskeleton, which was diminished in HeLa cells by inhibiting TGF- β signaling. This was consistent with a previous study, which demonstrated TGF- β 1-dependent actin cytoskeleton rearrangement through the activation of Rho GTPases, such as cdc42 and RhoA [24]. However, the effect of APE1 downregulation on actin cytoskeleton was observed to be different in HeLa and A549 cells. This suggests that, although APE1 downregulation commonly influences actin cytoskeleton organization, its consequence differs according to cell type. Our results where APE1 knockdown enhanced cellular motility in A549 cells (Fig. 4D), but not in HeLa cells (unpublished results), partly supports this assumption. The mechanism behind this inconsistency remains unknown, and must be investigated further. Furthermore, APE1 downregulation enhanced the motility of A549 cells (reduced by inhibited TGF- β signaling; Fig. 4D), which suggested that APE1 influences cellular motility via TGF- β 1 gene regulation. TGF- β is a major epithelial–mesenchymal transition (EMT)-inducing factor [25]. EMT induced by TGF- β is associated with the downregulation of E-cadherin [26] and actin cytoskeletal rearrangement [16], thereby increasing cellular motility. Therefore, promotion of cellular motility by APE1 downregulation could be associated with the induction of EMT through the production of TGF- β 1.

In conclusion, this study demonstrated that APE1 downregulation promoted the expression of TGF- β 1, causing the rearrangement of actin cytoskeleton. We also discovered that APE1 knockdown enhanced cellular motility in A549 cells, which was suppressed by the inhibition of TGF- β 1 signaling. We believe our study provides novel insights into elucidating the biological significance of APE1 function.

Conflict of interest

The authors declare no conflict of interest.

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