

DNA methyltransferase inhibitor suppresses fibrogenetic changes in human conjunctival fibroblasts

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Purpose: This study aimed to clarify the effects of a DNA methyltransferase inhibitor on fibrogenetic changes in human conjunctival fibroblasts (HConF).

Methods: HConF were pretreated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-Aza-dC) for 48 h. After one passage, the cells were treated with 5 ng/ml of transforming growth factor (TGF)- β 2 for 48 h, and the expression levels of α -smooth muscle actin (α -SMA), extracellular matrix proteins, and phosphorylated Smad3 were evaluated with western blotting. A fusion construct between the *COL1A2* promoter and the luciferase gene was introduced into the HConF after the first passage, and the construct's activity was detected via a luciferase reporter gene assay.

Results: TGF- β 2-induced upregulation of α -SMA was suppressed by pretreatment with 5-Aza-dC (0.1, 1.0, and 10 μ M) in a dose-dependent manner. Upregulation of type I collagen was also suppressed by 10 μ M 5-Aza-dC pretreatment. In contrast, 5-Aza-dC had no inhibitory effect on the expression of fibronectin or phosphorylated Smad3. However, *COL1A2* promoter activity was suppressed with 5-Aza-dC pretreatment.

Conclusions: In HConF, fibrogenetic changes were partly suppressed with a DNA methyltransferase inhibitor, suggesting an indirect inhibitory effect of the inhibitor on the *COL1A2* promoter in HConF.

Fibrogenetic changes in fibroblasts play significant roles in the molecular mechanisms of inflammatory diseases of the ocular surface, and in pathological changes after ocular surgery [1-7]. An excessive wound healing process can cause dry eye, corneal opacification, disturbed eye movement, and surgical failure after corneal or glaucoma filtration surgery. Therefore, it is important to clarify the molecular mechanisms of pathogenic fibrosis, and to control the wound healing process on the ocular surface. However, treatment-resistant cases of ocular disease are prevalent, resulting in severe visual loss despite intensive care.

DNA methylation controls gene transcription as an epigenetic factor, resulting in the regulation of various cell functions. The DNA methyltransferase (DNMT) family comprises DNMT1, DNMT2 (or TRDMT1), DNMT3a, and DNMT3b, which catalyze the transfer of a methyl group to DNA. Because DNMTs are involved in cell survival, cell proliferation, cell differentiation, and tissue fibrosis, they have been investigated as possible therapeutic targets for cancer and fibrogenetic diseases [8-12]. Some DNMT inhibitors, such as azacitidine and its deoxy derivative, decitabine, are clinically used for the treatment of myelodysplastic

syndrome and acute myeloid leukemia. Of these, decitabine, also known as 5-aza-2'-deoxycytidine (5-Aza-dC), has anti-fibrotic effects, which have been reported in various organs, including the lungs [13-17], kidneys [18,19], liver [20-22], heart [23,24], and trabecular meshwork [25]. In contrast, some studies have reported that 5-Aza-dC induces fibrotic changes in fibroblasts, including cardiac [26], pulmonary [27], and Tenon's capsule fibroblasts [28]. Thus, the effects of 5-Aza-dC on tissue fibrosis have been suggested to be context-dependent.

We report the effects of 5-Aza-dC on fibrogenetic changes in conjunctival fibroblasts. The induction of α -smooth muscle actin (SMA) and type I collagen by transforming growth factor (TGF)- β 2 was suppressed with 5-Aza-dC pretreatment. In contrast, the suppressive effect of 5-Aza-dC on the expression of fibronectin was limited. These effects are suggested to be mediated by secondary factors.

METHODS

Cell culture and treatment: Primary human conjunctival fibroblasts (HConF) were obtained from ScienCell Research Laboratories (Carlsbad, CA), and cultured according to the manufacturer's recommendations [29]. HConF at passages 3-4 were used in all experiments, and were pretreated with 5-Aza-dC (Sigma-Aldrich, St. Louis, MO) at 0.1, 1.0, or

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10 μ M once daily for 2 days. The cells were then passaged and analyzed.

Western blot analysis: After passage, the culture medium was changed to serum-free Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan) for 1 h before treatment with 5 ng/ml of TGF- β 2 (R&D Systems, Minneapolis, MN). After 48 h of treatment with TGF- β 2, the cells were lysed. Western blotting was conducted as reported previously [29,30]. Briefly, equal amounts of total protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in Novex Bis-Tris protein gels or Tris-acetate protein gels (Thermo Fisher Scientific, Waltham, MA), and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After blocking, the membranes were probed with primary antibodies, and finally, probed with the corresponding secondary antibodies. The primary antibodies included anti- α -SMA (1:1,000; Sigma-Aldrich), anti-fibronectin (1:1,000; Abcam, Cambridge, UK), anti-type I collagen (1:1,000; Abcam), anti-Smad3 (1:1,000; Cell Signaling Technology, Danvers, MA), anti-phospho-Smad3 (Ser423/425, 1:1,000; Abcam), anti-Akt (1:1,000; Cell Signaling Technology), and anti-phospho-Akt (Ser473, 1:1,000; Cell Signaling Technology) antibodies. The secondary antibodies included horseradish peroxidase (HRP)-linked anti-rabbit (1:2,500; Cell Signaling Technology) and sheep anti-mouse (1:20,000; GE Healthcare) antibodies. ECL Prime Western Blotting Detection Reagent (GE Healthcare) was used to visualize protein bands. Digital images were obtained using a Luminescent Image Analyzer (LAS-4000; Fujifilm, Tokyo, Japan). The band densities of each sample were normalized to that of β -actin.

Real-time RT–PCR: Total RNA was extracted from cultured HConF using NucleoSpin® RNA (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. RNA was reverse transcribed using Prime Script RT Master Mix (Takara Bio). Quantitative real-time reverse transcription PCR (RT–PCR) was performed in a 20 μ l reaction mixture containing 10 μ l of PCR master mix (TB Green Premix TaqII, Takara Bio), 0.4 μ M primer pairs, and 2 μ l of cDNA samples using a Step One Plus real time PCR system (Thermo Fisher Scientific). The thermal cycling conditions were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. All real-time PCR experiments used the primer of ribosomal protein S18 (RPS18, Takara Bio, sequence not disclosed) as control. The other primer sequences were as follows: *ACTA2* (Gene ID 59; OMIM 102620; sense sequence, 5'-GTG TTG CCC CTG AAG AGC AT-3' and antisense sequence, 5'-GCT GGG ACA TTG AAA GTC TCA-3'), *FNI* (Gene ID 2335; OMIM

135600; sense sequence, 5'-CGG TGG CTG TCA GTC AAA G-3', and antisense sequence, 5'-AAA CCT CGG CTT CCT CCA TAA-3'), and *COL1A1* (Gene ID 1277; OMIM 120150; sense sequence, 5'-GTG CGA TGA CGT GAT CTG TGA-3', and antisense sequence, 5'-CGG TGG TTT CTT GGT CGG T-3').

WST-8 assay: After the 5-Aza-dC treatment for 48 h, the cells were seeded in 96-well plates at the density of 1×10^4 cells per well, and incubated for 24 h. Then the cells were starved for 1 h, and treated with or without TGF- β 2 for 48 h. After 48-h treatment, we examined cell proliferation using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. The absorbance at 450 nm was measured as the live cell numbers using a microplate reader (Multiskan FC; Thermo Fisher Scientific) after incubation with 10 μ l CCK-8 solution for 2 h.

Cytotoxicity assay: Cytotoxicity was evaluated as reported previously [29]. Briefly, live cells were stained simultaneously with Hoechst 33,342 (Dojindo, Kumamoto, Japan) and propidium iodide (PI) according to the manufacturer's protocol. After 30 min, the cells were observed under a fluorescence microscope (model BZ 700; Keyence, Osaka, Japan), and the numbers of live cells (Hoechst-positive and PI-negative) were counted. As a positive control, cells were treated with 4 mM H₂O₂ for 2 h.

Lactate dehydrogenase assay: We examined cytotoxicity using Cytotoxicity LDH assay Kit-WST (Dojindo) according to the manufacturer's protocol. We evaluated the lactate dehydrogenase (LDH) activity under the same conditions as the WST-8 assay. The absorbance at 492 nm was measured as LDH activity using a microplate reader.

Luciferase assay: After 48 h of pretreatment with 10 μ M 5-Aza-dC, the cells were seeded the day before transfection in 6-well plates. Transfection of HConF was performed at 80% confluency using GeneJuice® Transfection Reagent (Merck Millipore, Billerica, MA) according to the manufacturer's protocol, as described previously [31]. Briefly, transcriptional activity was assessed with the transient transfection of a luciferase reporter gene fused to the *COL1A2* (Gene ID 1278; OMIM 120160) promoter. As an internal control, a plasmid containing *Renilla* luciferase (pRL-TK; Promega, Madison, WI) was cotransfected. At 24 h after transfection, the medium was changed to serum-free DMEM. After another 24 h, the cells were stimulated with 5 ng/ml of TGF- β 2 for 24 h, and luciferase activity was assessed.

Statistical analysis: All experiments were repeated at least four times. The results are presented as the mean \pm standard error of the mean. All data were analyzed with the

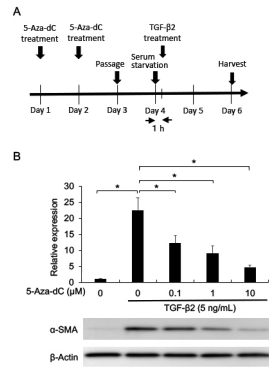


Figure 1. The effect of 5-aza-2'-deoxycytidine (5-Aza-dC) on α -SMA expression. **A:** Time course of the experiment. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. After passage, the cells were treated with transforming growth factor (TGF)- β 2 for 2 days, and then subjected to western blotting. **B:** The protein

bands were quantified, and the ratios of α -smooth muscle actin (SMA) to β -actin were calculated (n=5); *p<0.05. Bars indicate the standard error.

Tukey-Kramer multiple comparisons test. In all analyses, a p value of less than 0.05 indicated statistical significance.

RESULTS

The effect of 5-Aza-dC on α -SMA and the extracellular matrix: HConF were pretreated with 5-Aza-dC (0.1, 1.0, and 10 μ M) for 48 h. The cells were then passaged and treated with 5 ng/ml of TGF- β 2 for 48 h (Figure 1A). TGF- β 2 treatment induced the expression of α -SMA, fibronectin, and type I collagen (Figure 1B and Figure 2A,B). 5-Aza-dC suppressed TGF- β -induced α -SMA expression in a dose-dependent manner (Figure 1B). In contrast, its effect on the expression of fibronectin was not statistically significant (Figure 2A). Notably, treatment with 10 μ M 5-Aza-dC suppressed the

TGF- β -induced expression of type I collagen at basal levels (Figure 2B).

In addition to the expression of fibrogenic proteins, the mRNA expression levels of genes that code these proteins were analyzed. In agreement with the protein expression levels, the suppressive effects of 5-Aza-dC were prominent on *ACTA2* and *COL1A1*, whereas its effect on *FNI* was limited (Figure 3). Thus, TGF- β -induced fibrogenetic genes in HConF were partly suppressed with pretreatment with 5-Aza-dC.

The effect of 5-Aza-dC on cell proliferation: To assess the effect of 5-Aza-dC on the proliferation of HConF, the numbers of live and dead cells were counted. Treatment with 5-Aza-dC for 48 h decreased the numbers of live cells with or without TGF- β 2 in a dose-dependent manner (Figure 4A).

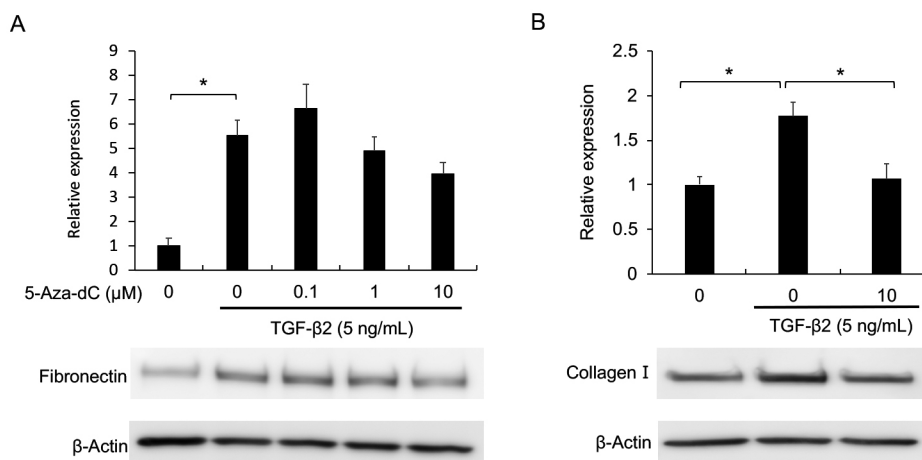


Figure 2. The effect of 5-Aza-dC on the extracellular matrix. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. After passage, the cells were treated with transforming growth factor (TGF)- β 2 for 2 days, and then subjected to western blotting. The protein bands were quantified, and the ratios of fibronectin (**A**) and type I collagen (**B**) to β -actin were calculated (n=5); *p<0.05. Bars indicate the standard error.

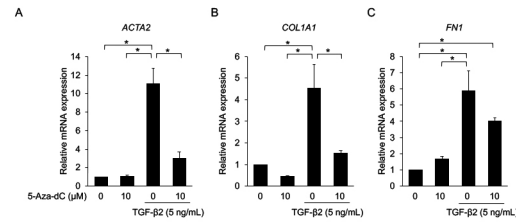


Figure 3. The effect of 5-Aza-dC on the expression of mRNA coding fibrogenic genes. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. After passage, the cells were treated with transforming growth factor (TGF)- β 2 for 2 days, and then subjected to real-time reverse transcription-PCR (RT-PCR). The expression levels of *ACTA2* (A), *FN1* (B), and *COL1A1* (C) were calculated (n=4); *p<0.05. Bars indicate the standard error.

Additionally, the WST-8 assay indicated the suppressive effect of 5-Aza-dC on TGF- β 2-induced cell proliferation (Figure 4B). To clarify whether the difference in the cell numbers was caused by cell death or suppressed proliferation, dead cells were visualized using PI staining and LDH assay. Even at the maximum dose (10 μ M), 5-Aza-dC did not induce cell death (Figure 5), suggesting that treatment with 10 μ M 5-Aza-dC suppressed cell proliferation without toxicity.

The effect of 5-Aza-dC on Smad signaling: Because 5-Aza-dC partly suppressed TGF- β -induced gene expression, we determined the levels of Smad3 expression and its phosphorylation in HConF. Treatment with TGF- β 2 for 48 h decreased Smad3 expression, and increased its phosphorylation (Figure 6). As a result, the ratio of phosphorylated Smad3 to total Smad3 was increased with TGF- β 2 treatment. Notably, these changes were not affected by 5-Aza-dC pretreatment.

To further investigate the effects of 5-Aza-dC on intracellular signaling, time-dependent changes in Smad3 and Akt at earlier periods (3, 6, and 24 h after treatment). As shown in Figure 7, the suppressive effect of TGF- β 2 on total Smad3 was observed at 24 h after treatment. At that time, the ratio of

phospho-Smad3 to total Smad3 was suppressed by 5-Aza-dC to some extent. However, the ratio of phospho-Akt on total Akt was not affected by the 5-Aza-dC treatment at any time point. These results suggest that the effect of 5-Aza-dC on TGF- β signaling was limited.

The effect of 5-Aza-dC on the promoter activity of type I collagen: To investigate whether 5-Aza-dC suppressed collagen expression by directly affecting the promoter of the type 1 collagen gene, we introduced a plasmid containing the promoter sequence of *COL1A2* into the HConF. Because 5-Aza-dC was absent at the time of plasmid induction (Figure 8A), the direct effect of 5-Aza-dC on the plasmid sequence was avoided. In this experiment, we tried two doses, 2.5 ng/ml and 5.0 ng/ml, of TGF- β 2, because the relative activity of the promoter is higher at the former dose. TGF- β 2 treatment statistically significantly activated the *COL1A2* promoter in HConF, and the TGF- β -induced activation of the *COL1A2* promoter was suppressed in HConF pretreated with 5-Aza-dC (Figure 8B, 8C), suggesting that 5-Aza-dC indirectly suppressed the TGF- β -induced expression of type I collagen.

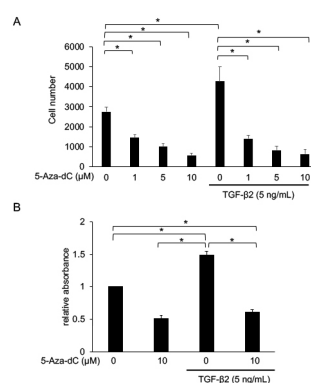


Figure 4. The effect of 5-Aza-dC on cell proliferation. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. The cells were then passaged and treated with transforming growth factor (TGF)- β 2 for 2 days. A: The cells were stained with Hoechst 33,342 and propidium iodide (PI). The

number of live cells (Hoechst-positive, PI-negative) was counted (n=4). B: The cells were subjected to the WST-8 assay (n=4); *p<0.05. Bars indicate the standard error.

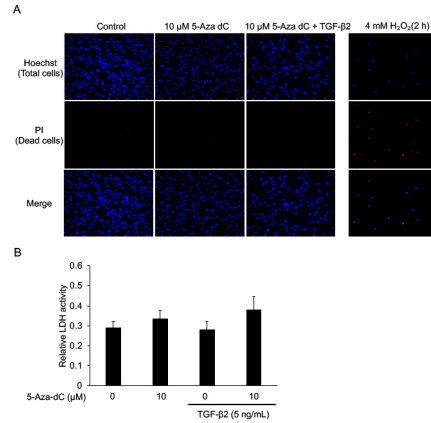


Figure 5. The effect of 5-Aza-dC on cell death. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. After passage, the cells were treated with transforming growth factor (TGF)-β2 for 2 days. **A:** The cells were stained with Hoechst 33,342 and PI. Cells stained with Hoechst and PI were considered dead cells (n=4). Cells treated with 4 mM H₂O₂ for 2 h were used as a positive control. **B:** The cells were subjected to lactate dehydrogenase (LDH) assay (n=4). Bars indicate the standard error.

DISCUSSION

In the present study, pretreatment with a DNMT inhibitor, 5-Aza-dC, prevented the TGF-β2-induced expression of α-SMA and type I collagen (Figure 1, Figure 2, and Figure 3), and the proliferation (Figure 4 and Figure 5) of HConF. However, its effect on the expression of fibronectin was limited (Figure 2 and Figure 3). Although the effects of 5-Aza-dC on tissue fibrosis were context-dependent, these results suggest that 5-Aza-dC had an antifibrosis effect on the conjunctiva for days (at least). To the best of our knowledge,

this study is the first to report the antifibrotic effects of this DNMT inhibitor on conjunctival fibroblasts.

Because the expression of α-SMA by fibroblasts is considered to indicate the transdifferentiation of cells into myofibroblasts, and subsequently, to a more fibrogenetic cell type, its expression level was evaluated as a marker of fibrosis in vivo and in vitro. In various tissues, including the lungs, kidneys, liver, and heart, 5-Aza-dC suppressed α-SMA expression [13,15,18,22,23], consistent with the results of the present study (Figure 1 and Figure 3). In the lungs, heart, and Tenon’s capsule, however, there have been controversial

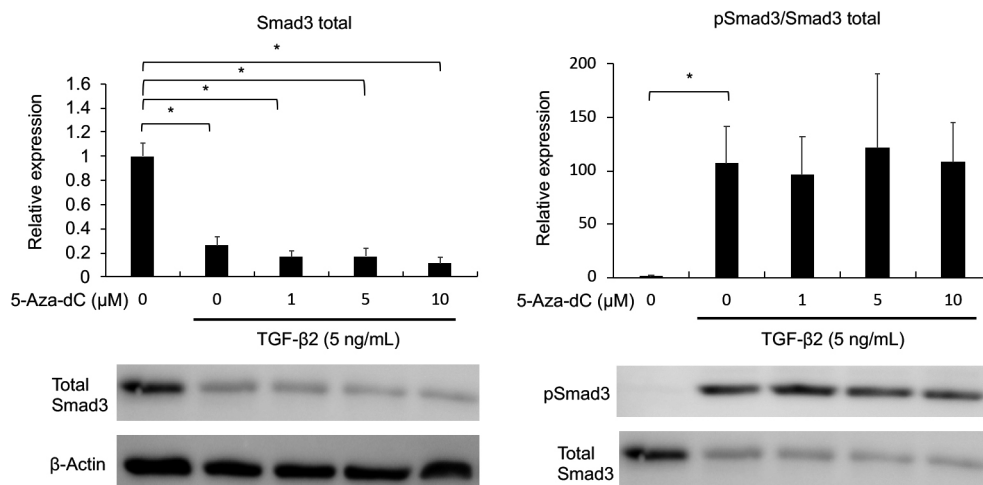


Figure 6. The effects of 5-Aza-dC on Smad3. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. The cells were then passaged, and treated with transforming growth factor (TGF)-β2 for 2 days. The cells were then subjected to western blotting. The protein bands were quantified, and the ratios of total Smad3 to β-actin (**A**), and phosphorylated Smad3 to total Smad3 (**B**), were calculated (n=4); *p<0.05. Bars indicate the standard error.

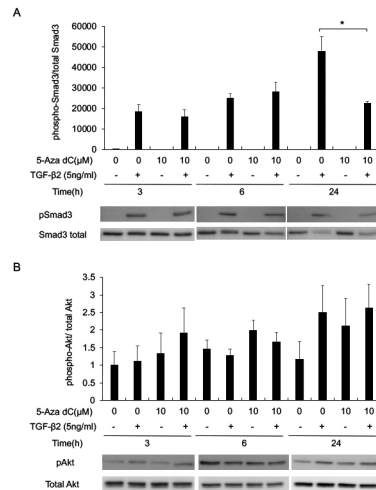


Figure 7. The effects of 5-Aza-dC on time-dependent changes in Smad3 and Akt. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. The cells were then passaged, and treated with transforming growth factor (TGF)-β2 for 2 days. The cells were then subjected to western blotting. The protein bands were quantified, and the ratios of phospho-Smad3 to total Smad3 (A), and phospho-Akt to total Akt (B), were calculated (n=3 and 4, respectively); *p<0.05. Bars indicate the standard error.

results showing that 5-Aza-dC increased α-SMA expression in fibroblasts [26-28]. Although the reason for this controversy is unclear, its effect might be dependent on the cell type, surrounding conditions, and/or manner of 5-Aza-dC treatment.

Type I collagen is a major component of the extracellular matrix, where it maintains tissue integrity. Notably, the ratio of type I collagen increased in keloid tissue [31], suggesting that type I collagen has significant roles in pathogenic wound healing [32]. Similar to its effect on α-SMA expression, 5-Aza-dC suppressed the expression of type I collagen in various tissues [13-16,20,22,23,25], but increased its expression under other conditions or in other cell types

[26,28]. In the present study, the TGF-β2-induced expression of type I collagen was suppressed to almost basal levels by pretreatment with 5-Aza-dC in HConF (Figure 2, Figure 3). Furthermore, 5-Aza-dC pretreatment suppressed the activity of the promoter of *COL1A2*, which encodes one of the fibers in type I collagen (Figure 8). Because the plasmid containing the promoter was introduced into cells after the washout of 5-Aza-dC during passage, the results suggest that 5-Aza-dC-induced changes in cellular properties secondarily suppressed the expression of type I collagen. Consistent with this possibility, 5-Aza-dC pretreatment did not affect Smad signaling.

Because DNA methylation by DNMTs suppresses gene expression, the direct effect of 5-Aza-dC involved the

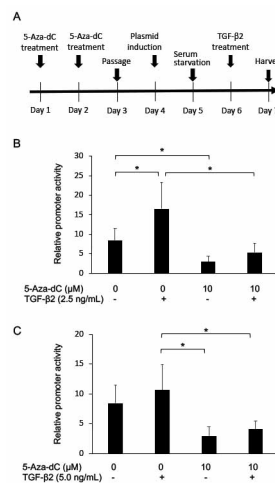


Figure 8. The effects of 5-Aza-dC on the promoter activity of *COL1A2*. A: The time course of the experiment. Primary human conjunctival fibroblasts were pretreated with 5-Aza-dC once daily for 2 days. The cells were then passaged, and plasmids containing the *COL1A2* promoter were introduced. After 24 h of serum starvation, the cells were treated with transforming growth factor (TGF)-β2 (2.5 ng/ml or 5.0 ng/ml) for 24 h, and subjected to a luciferase assay. B, C: The transcriptional activity was assessed

with the transient transfection of a luciferase reporter gene fused with the *COL1A2* promoter, and the activity relative to an internal control was calculated (n=4); *p<0.05. Bars indicate the standard error.

upregulation of the target genes. The suppressive effects of 5-Aza-dC on the expression of α -SMA and type I collagen (Figure 1, Figure 2, and Figure 3) might, therefore, be mediated by other molecules that were induced by the drug. In other cells and tissues, organ fibrosis has been related to gene methylation. For example, phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*; Gene ID 5728; OMIM 601728) was hypermethylated during liver fibrosis, and 5-Aza-dC treatment prevented the loss of PTEN expression, resulting in the inhibition of fibrosis [21]. Other antifibrosis genes, including *THY1* (Gene ID 7070; OMIM 188230), *Cyclooxygenase-2* (*PTGS2*; Gene ID 5743; OMIM 600262), Krüppel-like factor4 (*KLF4*; Gene ID 9314; OMIM 602253), *SFRP5* (Gene ID 6425; OMIM 604158), *MECP2* (Gene ID 4202; OMIM 300005), *RASSF1A* (Gene ID 11186; OMIM 605082), and *RASAL1* (Gene ID 8437; OMIM 604118), have been shown to be suppressed by DNA methylation during fibrosis [13,15,16,18-20,24,25,33], and antifibrosis miRNAs are also potential mediators of 5-Aza-dC suppression during the induction of fibrogenetic genes [14].

Fibronectin provides most of the provisional matrix in dermal wounds at early stages of tissue repair [34], so its expression might be differently controlled when compared with that of type 1 collagen. Consistent with this possibility, fibronectin expression was not significantly suppressed by 5-Aza-dC pretreatment in HConF (Figure 2, Figure 3). Although the effect of 5-Aza-dC on fibronectin expression has been investigated less intensively compared to type I collagen expression, several studies have reported its suppressive effect on fibronectin [35-37]. Conversely, 5-Aza-dC increased fibronectin expression in Tenon's capsule fibroblasts [28]. Thus, the effects of 5-Aza-dC on the expression of fibrogenetic genes are context-dependent; additional studies are required to clarify the molecular mechanisms that control these differences.

Proliferation of fibroblasts is essential for normal wound healing process, and is accelerated in abnormal tissue scarring. In the present study, 5-Aza-dC suppressed cell proliferation with or without exogenous TGF- β 2 (Figure 4, Figure 5), similarly to previous studies in lung, liver, and heart [17,20,22,24]. This effect may relate to the transdifferentiation into myofibroblasts, which are known to proliferate more than naïve fibroblasts, because 5-Aza-dC inhibits the transdifferentiation as described above. Even in the absence of exogenous TGF- β 2, there might be an intrinsic one that functions in the autocrine manner. There is another possibility that 5-Aza-dC upregulates genes that slow the cell cycle. Of interest, 5-Aza-dC helped the demethylation of *FOXD3* (Gene ID 27022; OMIM 611539), and thus, decreased

cell proliferation of ovarian cancer cells [38]. Similarly, the methylation of other genes, such as *p21* (*CDKN1A*; Gene ID 1026; OMIM 116899), *SFRP5*, *ECRG4* (Gene ID 84417; OMIM 611752), and *HIF2 α* (*EPAS1*; Gene ID 2034; OMIM 603349), was also reportedly related to cell proliferation [39-42]. Furthermore, 5-Aza-dC inhibits the growth of the lung fibroblasts in hyperoxia-induced neonatal bronchopulmonary dysplasia rats in vitro by demethylating the *P16* (*CDKN2A*; Gene ID, 1029; OMIM 600160) gene, resulting in cell cycle arrest [17]. Exact mechanisms of the inhibitory effect of 5-Aza-dC on cell proliferation in HConF remain unknown, and future studies are required to clarify them.

In conclusion, the present study shows the antifibrogenetic effects of 5-Aza-dC on HConF. Because 5-Aza-dC has been used to treat myelodysplastic syndromes and acute myeloid leukemia, the clinical application of 5-Aza-dC for fibrotic diseases of the ocular surface is a possibility. However, unknown molecular mechanisms involving the antifibrogenetic effects of 5-Aza-dC on HConF remain. Additional in vitro and in vivo studies using animal models are required before clinical trials.

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