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All-*trans* retinoic acid activates E-cadherin expression via promoter hypomethylation in the human colon carcinoma HCT116 cells

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ABSTRACT

All-*trans* retinoic acid (ATRA) inhibits the invasive and metastatic potentials of various cancer cells; however, the underlying mechanism is unclear. Here, we show that ATRA activated E-cadherin expression via promoter hypomethylation to facilitate Sp1 binding on its recognition sites in human colon carcinoma HCT116 cells. This effect was mediated by retinoic acid receptor-β2, as demonstrated by knock-down experiments using a specific siRNA. As a result, ATRA increased cell-to-cell interactions, reduced cell migration, and downregulated levels of Vimentin and Fibronectin in HCT116 cells. The present study thus provides the mechanism for the beneficial effects of ATRA in the treatment of metastatic human carcinomas.

1. Introduction

All-*trans* retinoic acid (ATRA), the predominant natural metabolite of vitamin A, is involved in many important biological processes, including vision, morphogenesis, differentiation, growth, metabolism, and cellular homeostasis [1]. In addition, ATRA is being increasingly included in both chemopreventive and therapeutical schemes for various tumoral diseases including colon cancer [2,3]. In general, ATRA is believed to inhibit carcinogenesis by blocking the promotion of initiated or transformed cells by three mechanism: induction of apoptosis, arrest of further growth of abnormal cells and induction of abnormal cells to differentiated back to normal [2]. Several studies also have reported that ATRA inhibits cancer cell invasion and metastasis in diverse model systems. For example, metastatic human renal carcinoma cells treated with ATRA grow in a clustering manner with tight cell-to-cell contacts [4]. ATRA also can reduce the invasive and metastatic potentials of breast cancer [5], gastric cancer [6], thyroid cancer [7], and colon cancer cells [8]. However, the mechanism by which ATRA blocks the late stages of carcinogenesis is largely unknown.

E-cadherin as a member of the cadherin superfamily of calcium-dependent transmembrane glycoproteins plays an essential role in normal physiologic processes such as development, cell polarity, and tissue morphology [9,10]. Alterations in E-cadherin expression or functions are thus common during carcinogenesis [10,11]. In general, aberrant cellular distribution of E-cadherin or repression of its expression is accompanied during epithelial-to-mesenchymal transition (EMT), an essential component of cancer progression to more aggressive phenotypes characterized by tumor dedifferentiation, infiltration, and metastasis [11,12]. On the other hand, restoration of E-cadherin expression induces mesenchymal-to-epithelial transition (MET) that enhances intercellular adhesion, inhibits tumorigenicity, and suppresses the invasiveness of carcinoma cells [13,14]. In the present study, we investigated whether ATRA activates E-cadherin expression to inhibit cancer cell invasion and metastasis. For this purpose, we first explored whether and how ATRA activates E-cadherin expression in human colon carcinoma HCT116 cells. Second, we attempted to prove that RAR-β2 mediates the potential of ATRA to activate E-cadherin expression. Lastly, we confirmed that ATRA actually increases cell-to-cell interactions, inhibits cell migration, and induces other MET markers in HCT116 cells as the outcomes of E-cadherin upregulation.

2. Materials and methods

2.1. Cell lines and transfection

HCT116 (a human colon cancer cell line, KCLB No. 10247) and HepG2 (a human hepatoblastoma cell line, KCLB No. 88065) were obtained from the Korean Cell Line Bank. HaCaT (a human keratinocyte cell line) was kindly provided by B. H. Yun (Pusan National University, Korea). For transient expression, 2×10^5 cells per 60-mm dish were transfected with 1 μg of appropriate plasmid(s) using WelFect-EX™PLUS (WelGENE) following the manufacturer's instructions. Cells were either mock-treated or treated with 5 μM ATRA (Sigma) and/or 5 μM 5-Aza-3′dC (Sigma) for 48 h unless otherwise stated.

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2.2. Luciferase reporter assay

2×10^5 cells per 60-mm diameter plate were transfected with 0.5 μg of E-cad-luc [15] or $(RARE)_3$-TK-luc [16]. To control for transfection efficiency, 0.1 μg of pCH110 (Pharmacia) containing the *Escherichia coli lacZ* gene under the control of the SV40 promoter was cotransfected as an internal control. At 48 h after transfection, luciferase assay was performed and the value obtained was normalized to the β-galactosidase activity measured in the corresponding cell extracts.

2.3. RNA interference

pSUPER RNAi system (OligoEngine), a vector system for expression of short interfering RNA plasmid-based RNA interference system that uses H1 RNA-based polymerase III promoter, was employed to knock-down specific gene expression. Based on the target sequence of RAR-β_2 (5′-TTA AGC AGA TGG CAC TGA GAA-3′) [17], siRNA inserts composed of both sense and antisense sequences separated by a central hairpin sequence were designed. The siRNA inserts were ligated into pre-cut pSUPER vector and positive clones were selected.

2.4. Western blot analysis

Cells were lysed in buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40) supplemented with protease inhibitors. Cell extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF, Amersham). Membranes were then incubated with antibodies against DNA methyltransferase 1 (DNMT1), Fibronectin, RAR-β, Sp1, Vimentin (Santa Cruz Biotechnology), E-cadherin (Calbiochem), γ-tubulin (Sigma) for 2 h at room temperature and subsequently with the appropriate horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (H + L)-HRP (Bio-Rad) and anti-rabbit IgG (H + L)-HRP (Bio-Rad) for 1 h at room temperature. The chemiluminescent ECL kit (Amersham) was used to visualize protein bands via the ChemiDoc XRS imaging system (Bio-Rad).

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's instruction. The sheared chromatin was immunoprecipitated with an antibody against Sp1 (Santa Cruz Biotechnology) and a negative control rabbit IgG (Santa Cruz Biotechnology). DNA released from the precipitated complexes was amplified by PCR using a pair of primers for the detection of E-cadherin promoter (forward, 5′-ACT CCA GGC TAG AGG GTC ACC-3′; reverse, 5′- CCG CAA GCT CAC AGG TGC TTT GCA GTT CC-3′) as described before [18].

2.6. DNA methylation analysis

Genomic DNA (1 μg) denatured in 50 μl of 0.2 N NaOH was modified by treatment with 30 μl of 10 mM hydroquinone (Sigma) and 520 μl of 3 M sodium bisulfite (pH 5.0; Sigma) at 50 °C for 16 h. For methylation-specific PCR (MSP), the modified DNA (100 ng) was amplified with *Taq* polymerase using MSP primer pairs for E-cadherin as described before [19]. For bisulfite DNA sequencing, modified genomic DNA was amplified by PCR using the primer set, E-cadherin forward 1 (5′-ATT TTA GGT TAG AGG GTT AT-3′) and E-cadherin reverse 1 (5′-CAA ACT AAA TAC TTT AC-3′) [19]. A 209-bp region (−179 to +30), encompassing 17 CpG sites in the E-cadherin regulatory region was subcloned into the pGEM-T Easy vector (Promega) and ten individual clones were sequenced per sample.

2.7. Immunofluorescence analysis

Cells grown to confluence on coverslips were fixed in 4% formaldehyde (15 min, room temperature) and then perforated in methanol (10 min, −20 °C), followed by incubation with a primary antibody against E-cadherin (Calbiochem; overnight, 4 °C) and then anti-mouse IgG-FITC (Santa Cruz Biotechnology; 1 h, room temperature). Slides were prepared on UltraCruz mounting medium (Santa Cruz Biotechnology) and visualized using Axioscope fluorescence microscope (Carl Zeiss).

2.8. Wound-healing assay

For the determination of cell migration rate, the classical scratch wound-healing assay was performed as described before [20]. Briefly, 1×10^6 cells were plated and cultured to create a confluent monolayer in 60-mm dish. After gently scratching with a pipette tip to produce a wound, cells were incubated in the presence or absence of 5 μM ATRA and/or 5 μM 5-Aza-2′dC for 24 h. Wound healing rate was determined by measuring closure rate of the wound.

2.9. Cell migration assay

Fast aggregation assay was performed as described previously [21]. Cells were dissociated with Hank's balanced salt solution (HBSS) with 0.01% trypsin and 1 mM $CaCl_2$ and washed twice in Ca^{2+}-free HBSS. The resulting cells (1×10^5) resuspended in 2 ml of HBSS containing 1 mM $CaCl_2$ were incubated for 30 min at 37 °C on a gyratory shaker. After incubation, the total particle number (single cells plus cell clusters) in each cell suspension was counted. The degree of aggregation was represented by the aggregation index N_{30}/N_0, where N_0 was the total particle number before incubation and N_t was the total particle number after incubation for 30 min [21].

2.10. Statistical analysis

The values indicate means ± S.D. from at least three independent experiments prepared in duplicate. The difference between the means of the treatment group and the control was assessed with the paired two tailed *t* test; difference was considered to be significant if $P < 0.05$.

3. Results

3.1. ATRA activates E-cadherin expression via promoter hypomethylation

First, we examined whether ATRA activates E-cadherin expression in colon carcinoma HCT116 cells. Protein levels of E-cadherin were upregulated by ATRA in HCT116 cells in a dose-dependent fashion, reaching to the maximum level at 5 μM (Fig. 1A). In addition, the promoter activity of E-cadherin was significantly upregulated by ATRA (Fig. 1B), suggesting that ATRA activates E-cadherin expression at the transcription level. As the E-cadherin promoter does not contain a RA response elements (RARE), ATRA cannot directly activate its expression and thus requires an alternative mechanism. According to our recent report, ATRA down-regulates levels of DNMT1, 3a, and 3b to lower total DNMT activity [22]. In addition, E-cadherin expression is frequently deregulated via DNA methylation in a variety of human cancers including colon cancer [10,23]. To investigate whether ATRA activates E-cadherin expression via modulation of DNA methylation, HCT116 cells were either mock-treated or treated with ATRA and/or a universal DNMT inhibitor, 5-Aza-2′dC. Both the protein level and promoter activity

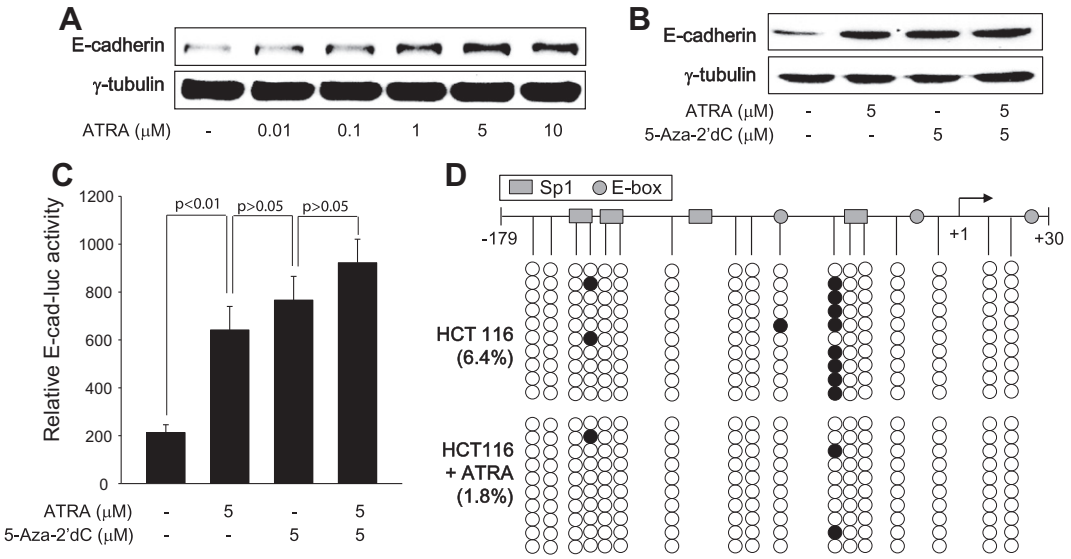

Fig. 1. ATRA induces expression of E-cadherin via inhibition of DNA methylation. (A) HCT116 cells were treated with an increasing concentration of ATRA for 48 h. Protein levels of E-cadherin and γ-tubulin were determined by Western blots. (B) HCT116 cells were transiently transfected with E-cad-luc [15] and then either mock-treated or treated with 5 μM ATRA and/or 5 μM 5-Aza-2′dC for 48 h followed by luciferase assay. (C) HCT116 cells were either mock-treated or treated with ATRA and/or 5-Aza-2′dC as above. (D) Bisulfite sequencing of the E-cadherin promoter region in HCT116 cells with or without ATRA treatment. The CpG sites in a 209 bp region (−179 to +30) of the E-cadherin promoter from ten different clones are shown as unmethylated (open circles) or methylated (filled circles). The positions of the Sp1 binding sites, E-boxes, and transcription initiation site are indicated.

of E-cadherin were similarly upregulated by treatment with either ATRA or 5-Aza-2′dC (Fig. 1B and C), indicating that E-cadherin expression can be activated via inhibition of DNA methylation in HCT116 cells. In addition, neither additive nor synergistic effect was induced by cotreatment with ATRA and 5-Aza-2′dC (Fig. 1B and C). These results suggest that ATRA activates E-cadherin expression via inhibition of DNA methylation.

To investigate whether ATRA actually inhibits DNA methylation of E-cadherin in HCTT116 cells, we performed bisulfite DNA sequencing analysis of genomic DNA isolated from HCT116 cells either mock-treated or treated with ATRA. DNA methylation was detected in a few CpG sites (6.4%) on the E-cadherin promoter (Fig. 1D). Interestingly, however, the CpG site near the fourth Sp1 binding site on the E-cadherin promoter was heavily methylated in the absence of ATRA. Treatment with ATRA inhibited DNA methylation of this CpG site and thus decreased the overall DNA methylation frequency to 1.8% (Fig. 1D). The DNA methylation frequency of E-cadherin promoter was also similarly reduced by treatment with 5-Aza-2′dC (data not shown).

3.2. ATRA enhances binding of Sp1 to E-cadherin promoter without affecting its protein level

We next examined whether the hypomethylation induced by ATRA affects Sp1 binding on the E-cadherin promoter. Neither ATRA nor 5-Aza-2′dC upregulated levels of Sp1 under the condition that they activated E-cadherin expression (Fig. 2A). According to ChIP analysis, treatment with either ATRA or 5-Aza-2′dC dramatically upregulated levels of Sp1 bound on the E-cadherin promoter (Fig. 2B). These results suggest that ATRA activates E-cadherin expression by inducing promoter hypomethylation to facilitate Sp1 recruitment.

3.3. RAR-β2 mediates the potential of ATRA to upregulate E-cadherin via promoter hypomethylation

According to previous reports, the anti-cancer potentials of ATRA are mostly mediated by RAR-β2 [24]. Therefore, it was examined whether RAR-β2 is responsible for the activation of E-cadherin expression by ATRA. ATRA substantially upregulated levels of RAR-β2 in HCT116 cells (Fig. 3A), resulting in elevation of its transcriptional activity (Fig. 3B). ATRA also induced similar effects in a human hepatoma cell line, HepG2 but not in a human keratinocyte cell line, HaCaT, in which the basal level of RAR-β2 and its transcriptional activity were relatively high (Fig. 3A and B). Interestingly, levels of E-cadherin in these cells were closely correlated with those of RAR-β2 and its transcriptional activity.

To provide more direct evidence that RAR-β2 mediates the potential of ATRA to activate E-cadherin expression, we attempted to knock-down RAR-β2 in the HCT116 cells treated with ATRA using a specific siRNA. As levels of RAR-β2 were knocked-down, the negative effect of ATRA on the level of DNMT1 was gradually abolished (Fig. 3C). Accordingly, the potential of ATRA to inhibit DNA methylation of E-cadherin was also impaired (Fig. 3D). As a consequence, the E-cadherin promoter became inaccessible to Sp1 (Fig. 3E), resulting in decrease in E-cadherin expression (Fig. 3C). Taken together, we conclude that RAR-β2 mediates the potential of ATRA to upregulate E-cadherin via promoter hypomethylation.

3.4. ATRA induces mesenchymal to epithelial transition of HCT116 cells

Lastly, we investigated whether the upregulation of E-cadherin by ATRA alters the metastatic properties of HCT116 cells. In the absence of ATRA, HCT116 cells exhibited relatively loose cell-to-cell contacts, leaving spaces between cells due to low levels of E-cadherin expression (Fig. 4A). In contrast, HCT116 cells displayed tighter and closer cell-to-cell interactions when levels of E-cadherin were upregulated by treatment with ATRA. To provide more direct evidence that ATRA increases cell-to-cell interactions, the cell aggregation ability of HCT116 cells was measured in the presence or absence of ATRA. As a result, the aggregation ability of HCT116 cells was significantly increased in the presence of ATRA (Fig. 4B).

It has been described that the reduced E-cadherin expression is also strongly correlated with enhanced cell migration *in vitro* and tumor progression *in vivo* [11]. Therefore, we examined whether ATRA inhibits cell migration via activation of E-cadherin expression. According to wound healing assay, HCT116 cells showed a highly migratory behavior, beginning to enter the wound after just

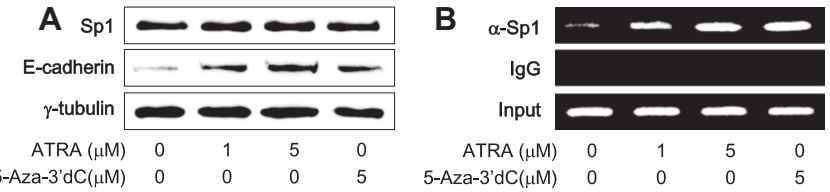

Fig. 2. ATRA enhances binding of Sp1 to E-cadherin promoter without affecting its protein level. HCT116 cells were either mock-treated or treated with ATRA and/or 5-Aza-2′dC as described in Fig. 1C. (A) Protein levels of Sp1, E-cadherin, and γ-tubulin were determined by Western blots. (C) ChIP assay was performed to determine levels of Sp1 bound to the positions of E-cadherin promoter.

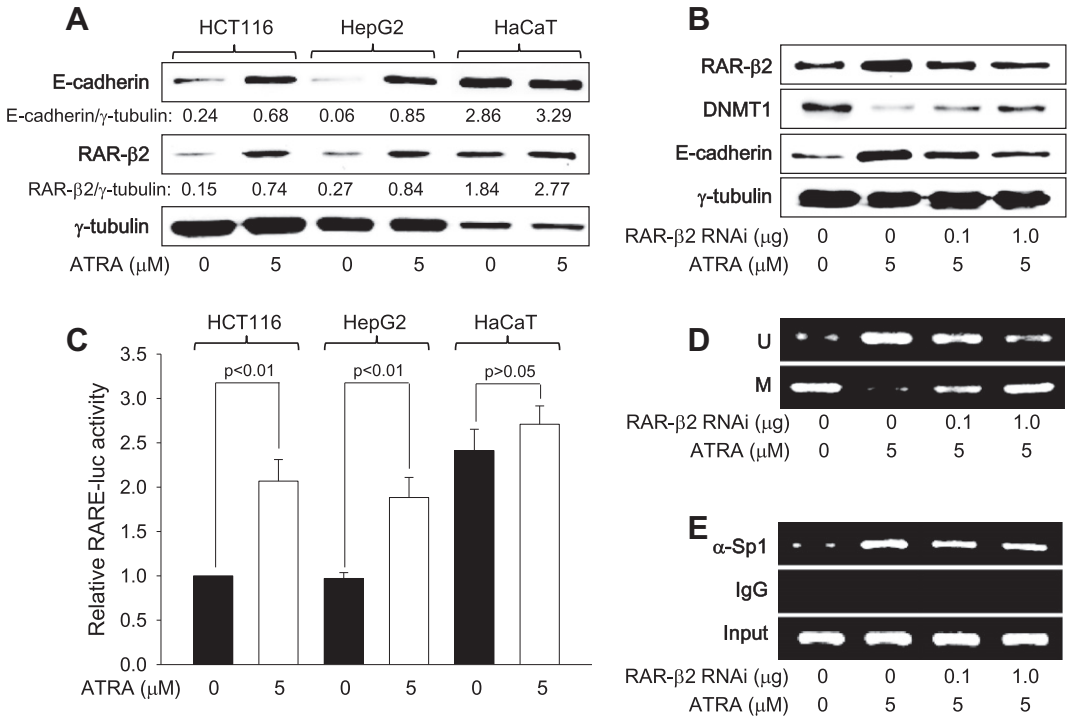

Fig. 3. RAR-β2 mediates the potential of ATRA to upregulate E-cadherin via promoter hypomethylation. Three different human cell lines, HCT116, HepG2, and HaCaT, were either mock-treated or treated with 5 μM ATRA for 48 h. Protein levels of E-cadherin, RAR-β_2, and γ-tubulin were determined by Western blots. Each band was quantified with the use of BIOPRORIL BIO 1D image analysis software (Vilber Lourmat). The values shown underneath the protein bands indicate the relative level of each protein to the loading control (γ-tubulin). (B) HCT116, HepG2, and HaCaT cell lines were transiently transfected with $(RARE)_3$-TK-luc [16] and then either mock-treated or treated with 5 μM for 48 h followed by luciferase assay. (C) HCT116 cells were transiently transfected with an increasing concentration of RAR-β_2 RNAi plasmid and then either mock-treated or treated with 5 μM ATRA for 48 h. Protein levels of RAR-β_2, DNMT1, E-cadherin, and γ-tubulin were determined by Western blots. (D) Genomic DNA purified from HCT116 cells prepared as above was modified by treatment with sodium bisulfite as described in Methods. MSP analysis was performed to determine whether the CpG sites within the E-cadherin promoter is methylated (M) or unmethylated (U). (E) ChIP assay was performed to determine the level of Sp1 bound to the positions of E-cadherin promoter in the HCT116 cells under the indicated conditions.

6 h postincision (data not shown). The wound surface was then almost completely colonized by HCT116 cells 24 h after the wound was made, whereas at this time about 60% of the incised surface still remained uncovered in the presence of ATRA (Fig. 4C).

The increased cell-to-cell interactions and reduced cell migration ability due to the elevated E-cadherin expression are critical phenotypes of MET [12]. In addition, ATRA also downregulated levels of mesenchymal markers such as Vimentin and Fibronectin under the condition that it upregulated levels of E-cadherin (Fig. 4D). Taken together, we conclude that ATRA induces MET of HCT116 cells via upregulation of E-cadherin expression. The universal DNMT inhibitor, 5-Aza-2′dC, produced the same effects on these MET phenotypes and markers in HCT116 cells (Fig. 4). In addition, neither additive nor synergistic effects were observed between ATRA and 5-Aza-2′dC, suggesting that they exert these potentials via a common pathway, i.e., inhibition of DNA methylation.

4. Discussion

Colon cancer, a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract, is the fourth most common cause of cancer death worldwide [25]. Death due to colon cancer is generally caused by hepatic metastasis of the primary tumor [26]. Interestingly, retinoid, a group of compounds consisting of vitamin A, its natural metabolites, and several synthetic compounds, have been shown to inhibit cancer metastasis. For example, ATRA decreases the invasive and metastatic potentials of breast cancer [5], gastric cancer [6], thyroid cancer [7], and colon cancer cells [8]. However, the mechanism(s) underlying these effects is poorly understood. We here demonstrate that ATRA activates E-cadherin expression in human colon carcinoma HCT116 cells to increase cell-to-cell interactions and thus inhibits their migration.

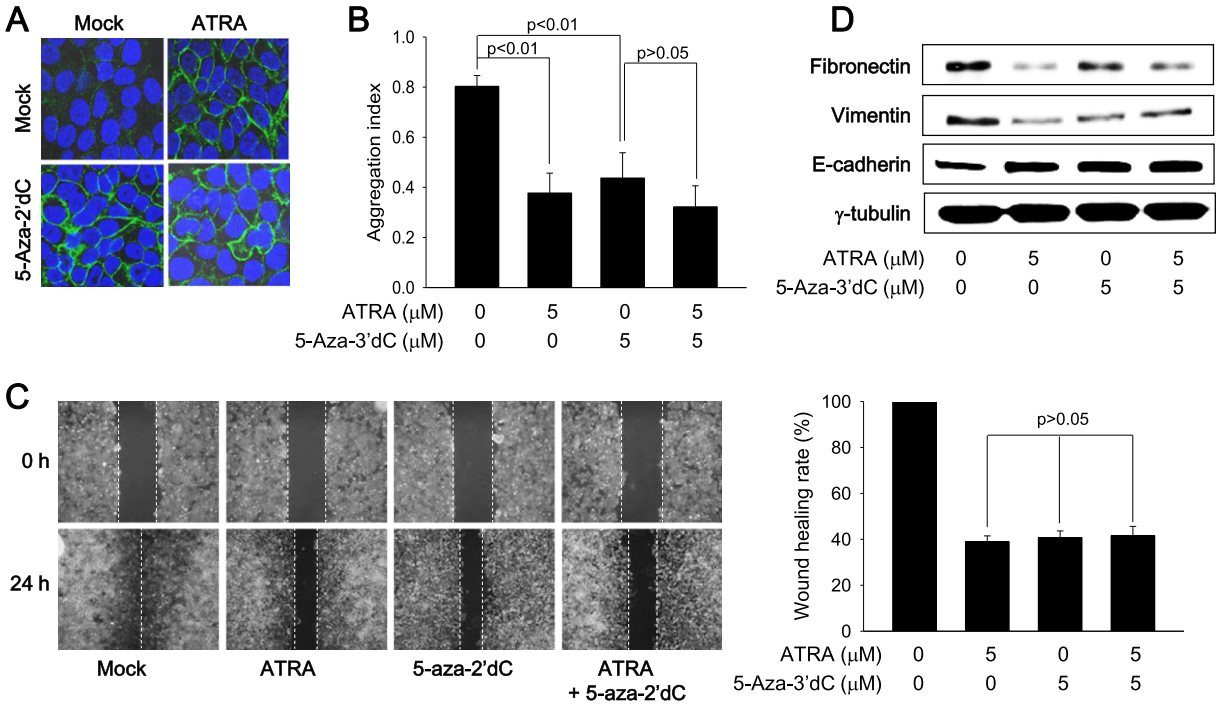

Fig. 4. ATRA induces mesenchymal to epithelial transition of HCT116 cells. HCT116 cells were either mock-treated or treated with ATRA and/or 5-Aza-2′dC as described in Fig. 1C. (A) Immunofluorescent images of HCT116 cells showing the localization and organization of E-cadherin (green) and nuclei (blue). (B) Fast aggregation assay of HCT116 cells was performed as described previously [21]. Cells dissociated by trypsin treatment to monocellular suspension were allowed to aggregate in suspension culture for 30 min. (C) The migratory behavior of HCT116 cells prepared as above was analyzed in an *in vitro* wound model. Photographs of the cultures were taken immediately after the incision (0 h) and after 24 h in culture. The dotted lines indicate the boundary of cells moved to wound areas. The wound-healing rate indicates the closure rate of the wound, i.e. the percentage of recovered area at 24 h postincision compared to the incision area at 0 h. (B) Western blot analysis was performed to measure levels of Fibronectin, Vimentin, E-cadherin, and γ-tubulin as a loading control.

In general, E-cadherin expression is known to be negatively regulated at the transcription level by transcriptional repressors. For example, the zinc-finger transcription factor Snail functions as a negative regulator by binding to the E-box motifs of E-cadherin promoter and recruits a transcriptional repressor complex containing mSin3A/HDAC [27,28]. However, E-cadherin expression in most human cancers seems to be dysregulated primarily via DNA methylation [23,27]. Interestingly, Rowling et al. [29] have demonstrated that rats treated with ATRA exhibits an overall decrease in endogenous methylation levels of hepatic DNA. In addition, ATRA down-regulates both expression and activity of DNMT during acute promyelocytic leukemia blast differentiation *in vitro* and *in vivo* [30]. Consistently, the present study demonstrates that ATRA induces promoter hypomethylation of E-cadherin gene and facilitates Sp1 binding to its recognition sites, resulting in activation of E-cadherin expression. Therefore, ATRA activates E-cadherin expression in a similar manner to that of 5-Aza-2′dC.

The effectiveness of ATRA as cancer chemotherapy is usually limited by the acquisition of retinoic acid resistance. Retinoic acid resistance occurs when tumors or tumor-derived cell lines cease spontaneously to respond to treatment with ATRA due to defect in RARα, RARβ, or RARγ induction in response to ATRA [31]. The HCT116 is one of such retinoic acid-resistant cell lines [32]. In contrast to ATRA-sensitive colon cancer cells like HCT-15, HCT116 cells are resistant to the cell growth inhibition by ATRA [33]. The present study however definitely showed that both the level and activity of RAR-β2 are normally induced by treatment with ATRA in this cell line. In addition, knock-down of RAR-β2 abolished the ability of ATRA to activate E-cadherin expression in the HCT116 cells. Therefore, the ATRA-resistance observed in the previous reports does not seem to be due to defective RAR-β2 expression in HCT116 cells. It is unknown whether defective expression of other subtypes or isoforms of RAR is responsible for the resistance of HCT116 cells to ATRA in cell growth inhibition. Considering that RAR-β_2 is a major executor of the anti-tumor potential of ATRA in a wide variety of cancers [16,24], another possibility also can be considered. According to our previous reports, p16 plays a major role as an executioner of ATRA-induced cell growth inhibition in several types of cell lines [34]. Interestingly, p16 is absent in HCT116 cells because of the frameshift mutation of the gene [35], suggesting that absence of p16 expression in this cell line is at least in part responsible for the resistance in cell growth inhibition by ATRA. In this respect, HCT116 cells seem to provide an ideal system to study the roles and action mechanism of ATRA during the late stages of carcinogenesis without affecting their growth. In addition, these findings should be valuable for the understanding of the beneficial effects of ATRA in the treatment of metastatic human carcinomas.

Acknowledgments

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