Methylated +58CpG site decreases *DCN* mRNA expression and enhances TGF-β/Smad signaling in NSCLC cells with high metastatic potential

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Abstract. Decorin (DCN) has been suggested to display an anti-metastatic role by antagonizing bioactive TGF- β in advanced human cancers. However, the epigenetic mechanisms by which defective expression of DCN causes cancer metastasis remain unclear. We focused on non-small cell lung cancer (NSCLC) cell lines with low metastatic potential (95C) and high metastatic potential (95D), which share a similar genetic background. Quantitative PCR and clonal bisulfite sequencing indicated that the methylation levels of the +58CpG site in the DCN 5'-UTR region was significantly higher in 95D cells with low expression of DCN mRNA compared to 95C cells with high expression of DCN mRNA. In silico prediction and ChIP assay showed a correlation between +58CpG site and AhR, which was reported as a transcriptional activator. Importantly, EMSA and luciferase reporter gene assays suggested that +58CpG methylation specifically diminished the recruitment of AhR to DCN 5'-UTR sequence and caused a reduction of approximately 50% in transcriptional activity. At baseline, 95D cells exhibited higher p-Smad3 levels and lower E-cadherin expression when compared with 95C cells. The demethylating agent 5-Aza significantly led to restoration of DCN expression, reduced level of p-Smad3, increased expression of E-cadherin in 95D cells. Taken together, we identified the methylated +58CpG in DCN 5'-UTR associated with reduced expression of DCN mRNA, and revealed that +58CpG methylation may

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be one of the mechanisms accounting for reduced recruitment of the transcriptional activator AhR to *DCN* 5'-UTR, and suggest that this mechanism promotes TGF- β /Smad signaling by enhancing the phosphorylation of Smad3, thereby downregulating E-cadherin in NSCLC cells with high metastatic potential.

Introduction

Lung cancer is the leading cause of cancer deaths in men and women worldwide (1). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. More than 50% of all patients diagnosed with lung cancer have advanced disease (2). Despite improvement in cancer treatment, the prognosis for NSCLC patients is still poor, with 5-year survival rate of approximately 10% (3). Since metastasis remains the cause of 90% of deaths from solid tumors (4), elucidating the mechanisms underlying lung cancer metastasis appeared to be very important.

Transforming growth factor β (TGF- β) is a ubiquitous and essential regulator of cellular and physiologic processes, such as cellular proliferation, migration, invasion and immunosurveillance (5,6). TGF- β is a double-edged sword in cancer development and progression. TGF- β signaling pathway plays a suppressive role in cell growth of early-stage tumors. On the other hand, it is recognized that metastasis of most tumor cells requires TGF- β activity, and the active TGF- β signaling became a promoter in late-stage tumors (7,8). TGF- β -induced phosphorylation of Smad3 is a vital step in the active TGF- β signaling (9) and Smad3 phosphorylation is required for lung epithelial-to-mesenchymal transition (10), which is a transition process that provides cancer cells with higher invasive and metastatic abilities (11).

Decorin (DCN) is a member of the extracellular matrix small leucine-rich proteoglycan family (12) and DCN regulates multiple processes in cancer cells (13,14). Accumulative evidence shows that DCN plays an inhibitory role in tumor growth and metastasis (15-19). This role can be played through DCN specifically binding to TGF- β and partially blocking the TGF- β signaling pathway, leading to growth suppression of distant tumors including lung cancer (20-22). Although there was evidence indicating that reduced expression of DCN was associated with lymphatic metastasis in lung cancer patients (23), the mechanisms by which the reduced DCN expression causes lung cancer metastasis appear to be elusive.

Our recent work supported the idea that DNA methylation could be epigenetically responsible for inactivation of tumor suppressor gene in NSCLC (24-26). Moreover, Doi *et al* and Irizarry *et al* suggested that DNA methylation alterations in individual CpG site may have a vital role in cancer progression (27,28). Therefore, based on the facts that *DCN* proximal promoter and 5'-UTR regions harbor several CpG sites and our present study showing that high-metastatic NSCLC cells presented lower expression of *DCN* mRNA compared with low-metastatic NSCLC cells, we hypothesized that individual CpG methylation epigenetically functions to affect *DCN* mRNA expression and TGF- β /Smad signaling in metastatic NSCLC cells.

Materials and methods

Cell culture and drug treatments. Low- (95C) and highmetastatic (95D) human NSCLC cell lines were purchased from Shanghai Institute of Biochemistry and Cell Biology and cultured in RPMI-1640 (Gibco-Invitrogen) with 5% fetal bovine serum (FBS, Gibco-Invitrogen) and incubated at 37°C in 5% CO₂. 95C and 95D cell lines share a similar genetic background and have different tumor metastatic potential (29,30). Cell treatment with DNA demethylating agent, 5-aza-2'-deoxycitidine (5-Aza) (Sigma-Aldrich), was performed according to the protocol described previously by us (25) with some modifications. Culture medium with 5% FBS was always used in the demethylation process. The concentration of 5-Aza was 10 μ M and the cells were harvested for analysis of DNA methylation on day 5. Drug treatment with 5-Aza is a currently accepted system to study effects of hypomethylation on gene expression (25).

NSCLC tissue samples. Lung carcinoma tissues were obtained after informed consent from patients with stage III or IV NSCLC in the First Affiliated Hospital of Soochow University. Stage III patients (n=5) had local lymph node metastasis ($T_{1-4}N_{1-2}M_0$), while stage IV patients (n=5) had distant organ metastasis ($T_{1-4}N_{1-2}M_1$). Pathological stages were determined according to the Revised TNM Staging System for Lung Cancer. NSCLC patients had received neither chemotherapy nor radiotherapy before tissue sampling. The tissue samples were snap-frozen and stored in liquid nitrogen before study. This study was approved by the Academic Advisory Board of Soochow University.

Transwell invasion and migration assays. For invasion assay, BioCoat Matrigel invasion chambers (BD Biosciences) with 8- μ m pore polycarbonate membranes were pre-treated with serum-free RPMI-1640 (Gibco-Invitrogen) medium at 37°C for 2 h. After removing the medium, we added 750- μ l medium with 10% FBS as chemoattractant to each lower chamber and then added 5x10⁴ cells in 250- μ l serum-free medium to each upper chamber. After 24-well plates were incubated at 37°C for 24 h, the inserts were removed and non-invasive cells on the upper surface were removed by a cotton swab. The invasive cells on the lower surface of the membrane were then fixed in 100% methanol for 15 min, air-dried, and stained with 0.1% crystal violet for 30 min. The cells were recorded with a digital camera. For migration assay, a similar procedure to the invasion assay was conducted, but omitting addition of Matrigel.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from the cells with or without treatment of 5-Aza using RNAiso Plus kit (Takara) according to the manufacturer's protocol. Synthesis of cDNA with reverse transcriptase was performed by M-MLV First Strand kit (Invitrogen). For analysis of mRNA expression of DCN, qRT-PCR analysis was carried out using Platinum[®] SYBR[®] Green qPCR SuperMix-UDG kits (Invitrogen) according to the kit manual. Real-time PCR was performed on a Bio-Rad CFX384[™] Real-Time PCR system. Primer sequences for DCN mRNA detection are as follows: 5'-CTGGTGGGCTGG CAGAGCATAA-3' (forward), 5'-TGTTGTGTCCAGGTGG GCAGAAGT-3' (reverse). The amplification conditions are described below: 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 66°C for 30 sec and 72°C for 1 min. As an internal control, β -actin mRNA was measured in the same reaction conditions. DCN mRNA expression levels were calculated following normalization to β -actin mRNA levels.

In silico prediction of functional CpG sites in DCN proximal promoter and 5'-UTR regions. A 1,230-bp sequence, including DCN proximal promoter (-200 to +1) and 5'-UTR (+1 to +1,030) regions (RefSeq: NC_000012.11 and NM_133503.2), was available for CpG sites selection. Methyl Primer Express[®] Software v1.0 (Applied Biosystems) and TFSEARCH (http:// mbs.cbrc.jp/research/ db/TFSEARCH.html) were used for the prediction of the putative functional CpG sites.

Clonal bisulfite sequencing. Genomic DNA was extracted from NSCLC cells with or without treatment of 5-Aza according to standard proteinase K digestion and phenol-chloroform extraction. The genomic DNA was modified with bisulfite using EpiTect Bisulfite kit (Qiagen) and subjected to clonal bisulfite sequencing. Briefly, after the bisulfite-treated DNA was purified by Qiagen Quickspin columns, *DCN* 5'-UTR region containing +58CpG was amplified from bisulfite-treated DNA by PCR and cloned into pMD19-T vector (Takara). Twenty clones obtained from each of the PCR products were randomly selected and sequenced for methylation level of +58CpG site. The primers designed for the bisulfite-PCR were as follows: 5'-TGTGTGTAAAATATTGTGTAAGGTT-3' (forward) and 5'-AATACTACTTTCTCCCTCTTCTCA-3' (reverse).

Western blot analysis. Cells with or without treatment of 5-Aza were lysed in RIPA buffer with protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich) and centrifuged; cell lysates were resuspended and denatured in SDS buffer, and then fractionated by SDS-PAGE electrophoresis. Proteins were transferred to nitrocellulose membranes (Millipore) and blocked with BSA/TBST buffer. Membranes were incubated with primary antibodies overnight before

incubation with the corresponding HRP-conjugated secondary antibodies. Detection was performed using ECL kit (Pierce, Rockford, IL, USA). All experiments were performed in triplicate. Densitometry values were determined using ImageJ 1.46r software (NIH). Densitometry values for p-Smad3 level were normalized to total Smad3, while E-cadherin and AhR was normalized to β -actin. Antibodies employed in the analysis were: anti-p-Smad3 and anti-Smad3 (CST), anti-E-cadherin (Abcam), anti-AhR and anti- β -actin (Santa Cruz), and antimouse and anti-rabbit secondary antibodies (Santa Cruz).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using EZ-ChIP[™] Chromatin Immunoprecipitation kit (Upstate). Cells grown on 15-cm dishes were fixed with 1% formaldehyde for 10 min at room temperature, and the cross-linked cells were washed twice with phosphate-buffered saline (PBS), and were scrapped in PBS buffer with protease inhibitors. The cell nuclei were collected by centrifugation of 700 x g at 4°C and resuspended in SDS lysis buffer with protease inhibitors, sonicated in ice and the cross-linked DNA was sheared to 200-1,000 bp, and the insoluble material was removed by centrifugation at 13,000 x g for 10 min. Then 100 μ l of the supernatant was diluted in 900 μ l buffer with protease inhibitors, and incubated with Protein G Agarose for 1 h at 4°C. After brief centrifugation, 10 μ l (1%) of the supernatant was saved as the Input at 4°C, and the remaining was used for the immunoprecipitation and was incubated with 5 μ g anti-AhR antibody (Santa Cruz) overnight at 4°C. Rabbit IgG antibody was used as a negative control. Antibody/ chromatin complexes were incubated with Protein G Agarose for 1 h at 4°C, and then Protein G Agarose-antibody/chromatin complex was collected by brief centrifugation. After washing followed by centrifugation, we eluted the complex and the Input to obtain protein-DNA complexes. De-crosslinking was performed to free DNA by incubating in NaCl, RNase A, EDTA and proteinase K, and DNA purification was conducted using spin columns. The ChIP DNA fragments were subjected to PCR, which amplified DCN 5'-UTR sequence containing +58CpG (positions +2 to +115). Specific ChIP primers used for PCR amplification were as follows: 5'-GAATAATAAGACACGCCCTGAAG-3' (forward) and 5'-CACGTTGCTCTTACCTCTTTTAA-3' (reverse).

Electrophoresis mobility gel shift assay (EMSA). Nuclear extracts were prepared from NSCLC cells using the ProteoJET[™] Cytoplasmic and Nuclear Protein Extraction kit (Fermentas). The cell nuclear extracts and synthetic doublestranded and 5' biotin-labeled oligonucleotides corresponding to the DCN +58 C (unmethylated) and +58 5mC (methylated) sequences (5'-CAAGCACGCAAAACAAATTG-3' and 5'-CAAGCA5mCGCAAAACAAATTG-3', respectively. The binding sequences of transcriptional factors AhR/Ar (underlined) were incubated at 25°C for 20 min using the LightShift Chemiluminescent EMSA kit (Pierce). Each reaction was performed in a total volume of 20 μ l containing 20 fmol labeled probe, 2 μ g nuclear extract and 50 ng Poly (dI · dC). The reaction mixture was separated on 6% non-denaturing PAGE gel, and the products were transferred to positivecharged nylon membrane and then detected by stabilized streptavidin-horseradish peroxidase conjugate (Pierce). For each competition assay, 200-fold unlabeled +58 C and +58 5mC probes were added to the reaction mixture before the addition of biotin-labeled probes.

Construction of luciferase reporter plasmids containing methylated +58CpG transfection and luciferase assays. Two fragments of interest containing the unmethylated and methvlated +58CpG (5'-CAAGCACGCAAAACAAATTG-3' and 5'-CAAGCA5mCGCAAAACAAATTG-3', respectively) were directly synthesized and then cloned into the pGL3-Basic luciferase vector (Promega). Subsequently, the constructs carrying the unmethylated or methylated +58CpG were transiently transfected into cells using Lipofectamine 2000 (Invitrogen) and Renilla pRL-TK construct (Promega) was co-transfected as a normalizing control. Twenty-four hours later, luciferase activity was measured by the Dual-Luciferase Reporter Assay kit (Promega) on a TD20/20 Luminometer (Turner Designs). Each experiment was performed in triplicate. Results are expressed as relative luciferase activities, which are obtained following normalization to Renilla luciferase activities.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed to determine expression levels of DCN protein from cell culture supernatants using Human Decorin ELISA kit (RayBio). Cell culture supernatants were extracted and pipetted to each well according to the manufacturer's instructions. After adding the ELISA Stop Solution to each well, we read immediately the absorbance at 450 nm on ELx800[™] Microplate Reader (BioTek). Each determination was performed in triplicate.

Statistical analysis. Results are represented as the means \pm standard deviations (SD) for mRNA or protein expression. Comparisons between two groups were performed with Student's t-test for continuous variables or with χ^2 test for categorical variables. Statistical differences were considered to be significant at P<0.05. All statistical analyses were performed using GraphPad Prism 5.02 software.

Results

DNA methylation is involved in DCN mRNA expression. First, we conducted transwell assay to confirm that 95D cells have higher migration and invasion abilities than 95C cells (Fig. 1A), the phenotype differences between the two cell lines are consistent with those reported previously (29,30). Thus, we examined the expression of DCN mRNA in low- (95C) and high-metastatic (95D) human NSCLC cell lines using qRT-PCR analysis, and found that DCN mRNA expression in 95D cells was lower by far than that in 95C cells (P<0.01) (Fig. 1B). After treatment of demethylating agent 5-Aza on 95D cells expressing low DCN mRNA, DCN mRNA expression was increased by ~10-fold (P<0.001) (Fig. 1C), suggesting the involvement of DNA methylation in DCN mRNA expression.

In silico prediction of functional CpG sites. Using Methyl Primer Express[®] software and TFSEARCH, we identified that one putative functional CpG site at position +58 in *DCN*



Figure 1. Expression of *DCN* mRNA in 95C and 95D cells, and in 95D cells with or without treatment of 5-Aza. (A) Transwell assay for migration and invasion abilities of 95C and 95D cells. (B) qRT-PCR analysis of *DCN* mRNA expressed in 95C and 95D cells. *DCN* mRNA levels were expressed as a relative index normalized against β -actin. (C) Expression of *DCN* mRNA in 95D cells with or without treatment of 5-Aza (10 μ M). Data presented are the mean \pm SD from three independent experiments, and each was done in triplicate. *P<0.05; **P<0.001; ***P<0.001.

5'-UTR, close to the transcription start site, was predicted to locate within a transcription factor AhR/Ar-binding sequence (5'-AAGCA<u>CG</u>CAAAACAAA-3') (Fig. 2A). Here we designate this putative functional CpG site as +58CpG. This prediction provides support for the notion that methylation of +58CpG may functionally affect *DCN* mRNA expression.

Methylation level of +58CpG is higher in 95D than 95C cells, and was associated with DCN mRNA expression in metastatic NSCLC. To test whether methylation level of +58CpG affects expression of DCN mRNA, we performed clonal bisulfite sequencing to compare the differences between 95C and 95D cells. As a result, we observed that the methylation level of +58CpG was significantly higher in 95D cells expressing low DCN mRNA than 95C cells expressing high DCN mRNA (95±5 vs.65±10%,P<0.01) (Fig. 2B-D). Comparably, the results obtained in tumor tissues suggested that methylation level of +58CpG correlates inversely with DCN mRNA expression in metastatic NSCLC tissues (Fig. 3).

AhR is recruited to DCN 5'-UTR region containing +58CpG. Aryl hydrocarbon receptor (AhR) was reported to be a transcriptional activator (31,32) and +58CpG was *in silico* predicted to be harbored in AhR/Ar-binding site at DCN 5'-UTR region (Fig. 2A), leading us to consider the possibility that AhR can bind to *DCN* 5'-UTR region containing +58CpG. First, we performed western blot analysis and found that AhR was positively expressed in both cell types, and AhR expression was lower in 95D than 95C cells (Fig. 4A). Subsequently, ChIP analysis using anti-AhR antibody showed that *DCN* 5'-UTR sequence containing +58CpG (positions +2 to +115) was bound by AhR in 95C cells, but not in 95D cells (Fig. 4B), indicating that there is a correlation between +58CpG and AhR, and also suggesting that high methylation level of +58CpG (Fig. 2D) may be one of the mechanisms accounting for reduced recruitment of AhR to *DCN* 5'-UTR region in 95D cells.

Methylation of +58CpG decreases binding ability of transcriptional factors to DCN 5'-UTR region. Next, we performed EMSA to determine whether methylated +58CpG can affect the ability of binding to transcriptional factors including AhR/Ar from the nuclear extracts. As illustrated in Fig. 4C, although both the probe containing unmethylated +58CpG and the probe containing methylated +58CpG may combine with specific DNA-binding proteins from 95D cells, the unmethylated probe has stronger binding ability than the methylated probe. This effect was consistently observed in the EMSA experiments, suggesting that the methylated +58CpG can decrease the ability of binding of transcriptional factors, including AhR/Ar, to DCN 5'-UTR region.



Figure 2. Schematic presentation of CpG site distribution in *DCN* proximal promoter and 5'-UTR and methylation level of one putative functional CpG site in 95C and 95D cells. (A) A map of CpG site distribution in *DCN* proximal promoter (-200 to +1) and 5'-UTR (+1 to +1,030) regions with a total length of 1,230-bp. There are in total 10 CpG sites, each of which is represented by a black vertical line and the thick black vertical line represents the putative functional +58CpG that is predicted to be harbored in AhR/Ar-binding site. The transcriptional start site (TSS) is indicated as +1 and the translation initiation site is denoted as +1031 (ATG). (B) Schematic summary for clonal bisulfite sequencing of +58CpG site. Methylation analysis was performed in 20 clones for 95C and 95D cells. Each number represents a single clone, and open or filled circles represent unmethylated or methylated +58CpG, respectively. (C) Representative sequences showing the presence or absence of methylation at +58CpG site. Unmethylated nucleotide C is converted to T after bisulfite treatment, but methylated C remains unchanged. (D) Summary of +58CpG methylation level in 95C and 95D cells. **P<0.01.

Methylation of +58CpG significantly inhibits luciferase reporter gene transcriptional activity. To further determine whether methylation of +58CpG alters *DCN* transcriptional activity by de-recruiting the transcriptional activator AhR, we therefore constructed luciferase reporter vectors containing the corresponding unmethylated and methylated fragments (AhR/Ar-binding sequence) and transiently transfected them into 95D cells. As illustrated in Fig. 4D, the methylated +58CpG caused a reduction of \sim 50% in transcriptional activity compared with the unmethylated +58CpG (P<0.01). Taken together with EMSA assay, the results suggest that methylation of +58CpG may repress *DCN* mRNA expression by diminishing the recruitment of AhR to *DCN* 5'-UTR region.

DCN inhibits phosphorylation of Smad3 and enhances *E-cadherin expression in metastatic NSCLC cells.* DCN was reported to prevent TGF- β -induced inhibition of lung morphogenesis (33), leading us to explore the effect of DCN on TGF- β /Smad signaling. Thus, we first used ELISA assay to determine the expression of DCN protein in cell culture



Figure 3. Methylation level of +58CpG and expression of *DCN* mRNA in metastatic NSCLC tissues. (A) +58CpG methylation level in stage III and IV NSCLC tissues. (B) qRT-PCR quantification of *DCN* mRNA expression in stage III and IV NSCLC tissues. All data are shown as the mean \pm SD. *P<0.05; **P<0.01.

supernatants, and found that DCN protein expression in 95D was significantly lower than that in 95C (P<0.001) (Fig. 5A). Then we used 95C and 95D cell lines to detected the level of phosphorylated Smad3 (p-Smad3), which is an indispensable downstream effector in canonical TGF-β/Smad signaling (9). At baseline, the level of p-Smad3 was higher in 95D expressing low DCN (0.79±0.07) than that in 95C expressing high DCN (0.41±0.05) (P=0.028, Fig. 5B). Correspondingly, 5-Aza treatment significantly restored expression of DCN protein in 95D cell culture supernatants (P<0.01) (Fig. 5C) and decreased the level of p-Smad3 in 95D cells (0.37±0.10 vs. 0.55±0.10; P=0.010) (Fig. 5D), suggesting that demethylation-induced restoration of DCN (Fig. 5C) antagonizes bioactive TGF-β (33), leading to a reduction in the p-Smad3 level and an inhibition of TGF-β/Smad signaling.

DCN-mediated inhibition of colorectal cancer migration is associated with E-cadherin (13,15-19,34). Here we examined expression of E-cadherin in 95D expressing low DCN and 95C expressing high DCN, and found that E-cadherin expression was significantly lower in 95D cells (0.25 ± 0.05) than 95C cells (0.73 ± 0.07) (P=0.016) (Fig. 5B). Correspondingly, 5-Aza treatment significantly increased expression of E-cadherin in 95D cells (0.70 ± 0.07 vs. 0.48 ± 0.10 ; P=0.013) (Fig. 5D). The results suggest that DCN may enhance expression of E-cadherin in metastatic NSCLC cells.





Figure 4. ChIP analysis for the involvement of AhR in binding to +58CpG site of *DCN* 5'-UTR, EMSA and luciferase reporter gene analyses of transcription factor-binding site containing +58CpG in *DCN* 5'-UTR region. (A) Western blot analysis for expression of AhR protein in 95C and 95D cells. AhR was positively expressed in both cells and AhR expression was lower in 95D cells than 95C cells. (B) 95C and 95D cells were subjected to ChIP assay in which 5 μ g anti-AhR antibodies were used. Presence of *DCN* 5'-UTR sequence containing +58CpG (positions +2 to +115) prior to immunoprecipitation was confirmed by PCR (1% Input), and IgG antibody was used as a negative control. PCR products were separated on 2.0% agarose gels with ethidium bromide staining. Data are representative of three independent experiments. (C) EMSA assay was completed with biotin-labeled oligonucleotide probes and cell nuclear extracts from 95D cells. Lanes 1 and 4 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without nuclear extracts; lanes 2 and 5 represent the mobilities of the labeled probes without



Figure 5. Level of *DCN* protein, p-Smad3 and E-cadherin expression in 95C cells and 95D cells with or without treatment of 5-Aza. (A) ELISA assay for DCN protein expressed in 95C and 95D cell culture supernatants. Data presented are the mean \pm SD from three independent experiments, and each was done in triplicate. ***P<0.001. (B) Western blot analysis for the levels of p-Smad3, total Smad3 and E-cadherin proteins in 95C and 95D cells. Protein expression was determined via densitometry. Densitometry values for p-Smad3 level were normalized to total Smad3 (t-Smad3), while E-cadherin was normalized to β -actin. Values for each protein are shown above the lanes. (C) ELISA assay for DCN protein expressed in culture supernatants of 95D cells with or without treatment of 5-Aza (10 μ M). Data presented are the mean \pm SD from three independent experiments, and each was done in triplicate. ***P<0.001. (D) Western blot analysis for the levels of p-Smad3 and E-cadherin proteins in 95D cells with or without treatment of 5-Aza (10 μ M). Data presented are the mean \pm SD from three independent experiments, and each was done in triplicate. ***P<0.001. (D) Western blot analysis for the levels of p-Smad3 and E-cadherin proteins in 95D cells with or without treatment of 5-Aza (10 μ M). Protein expression was determined via densitometry. Densitometry values for p-Smad3 level were normalized to t-Smad3, while E-cadherin was normalized to β -actin. Values for each protein are shown above the lanes.

Discussion

In this study, we have identified the methylated +58CpG in *DCN* 5'-UTR associated with reduced expression of *DCN* mRNA in high-metastatic NSCLC cells. Our findings reveal that +58CpG methylation may be one of the mechanisms accounting for reduced recruitment of transcriptional activator AhR to *DCN* 5'-UTR, and suggest that this mechanism promotes TGF- β /Smad signaling by enhancing phosphorylation of Smad3, thereby downregulates E-cadherin in high-metastatic NSCLC cells.

Compelling evidence has shown that DCN contributes to inhibition of tumor growth and metastasis (13,15-19,34). Goldoni *et al* reported that DCN can display an anti-metastatic role in breast cancer (16). Bi *et al* demonstrated that *DCN* may act as a tumor suppressor gene (15) and mediate inhibition of colorectal cancer growth and migration (13). Among soft tissue tumor patients with recurrent or metastatic lesions, lower levels of DCN were expressed in secondary lesions than those in primary lesions (17). Decreased expression of DCN was found to be significantly associated with lymphatic metastasis of lung cancer patients (23). In the present study, we focused on low- (95C) and high-metastatic (95D) NSCLC cells, which were more genetically similar to each other and could be an ideal model for cancer metastasis (29,30), and found that *DCN* mRNA was significantly lower in 95D cells compared with that in 95C cells, supporting the idea that *DCN* was involved in metastasis of malignant tumors, including lung cancer. Additionally, demethylating drug 5-Aza restored *DCN* mRNA expression in 95D cells, suggesting the involvement of DNA methylation in *DCN* expression. Treatment with 5-Aza is a currently accepted system to study effects of demethylation on gene expression, albeit this compound is recognized to be capable of inducing other cellular effects (25).

To figure out the epigenetic mechanisms underlying reduced *DCN* mRNA expression in high-metastatic NSCLC cells, we examined DNA methylation level of +58CpG that was predicted to locate within AhR/Ar-binding sequence of 5'-UTR region. Based on the evidence that AhR/Ar is reported to be a transcriptional activator (31,32), our findings support the idea that +58CpG plays a role in reducing *DCN* mRNA expression by a methylation mechanism in high-metastatic 95D cells. More recently, DNA methylation was recognized to suppress epigenetically gene expression via preventing the binding of TF to methylated CpG dinucleotides within TF-binding sites (35-37). This is substantiated by our ChIP, EMSA and luciferase experiments, which showed that methylated +58CpG can decrease its ability of binding to AhR and lead to a reduction in transcriptional activity. The results shed light on the important role of +58CpG methylation in NSCLC metastasis, and supported the notion that individual CpG methylation plays a vital role in cancer progression (27,28).

Given that DCN can cause growth inhibition of tumor cells including NSCLC by blocking TGF- β signaling (20-22), we are inspired to evaluate the effect of DCN expression on TGF- β / Smad signaling during NSCLC progression. To reach this goal, here we examine the level of p-Smad3, which is essential for activating canonical TGF- β /Smad signaling (9), and the expression of E-cadherin, which was reported to correlate with DCN-mediated inhibition of colorectal cancer migration (13), in 95C expressing high DCN and 95D expressing low DCN. Our results suggest that DCN inhibited phosphorylation of Smad3 and enhanced E-cadherin expression in metastatic NSCLC cells. Interestingly, demethylating drug 5-Aza treatment significantly decreased the level of p-Smad3 in 95D cells, suggesting that the blocking of TGF- β /Smad signaling may be caused by demethylation-induced restoration of DCN. This is consistent with previous data that DCN can antagonize bioactive TGF- β during lung growth and differentiation (33). Moreover, demethylation-induced restoration of DCN via 5-Aza treatment led to an increased E-cadherin in 95D cells. The results support the notion that TGF- β /Smad signaling is a promoter in late-stage tumors, including NSCLC (7,8,11,38), and DCN-mediated inhibition of colorectal cancer migration is associated with E-cadherin (12-14).

In conclusion, we identified the methylated +58CpG in DCN 5'-UTR associated with reduced expression of DCN mRNA, and revealed that +58CpG methylation may be one of mechanisms accounting for reduced recruitment of transcriptional activator AhR to DCN 5'-UTR, and suggest that this mechanism promotes TGF- β /Smad signaling by enhancing phosphorylation of Smad3, thereby downregulates E-cadherin in high-metastatic NSCLC cells.

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