Modulation of E-cadherin expression by K-Ras; involvement of DNA methyltransferase-3b

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Introduction

Ras proteins (Ha-Ras, N-Ras and K-Ras) serve as points for diverse extracellular signal-stimulated pathways regulating proliferation and differentiation (1–3). Mutant Ras proteins are locked in persistently activated guanosine triphosphate-bound state and confer essential features of malignancy such as cell cycle progression, motility, protection against apoptotic stimuli and angiogenesis. However, k-ras is one of the most frequently mutated genes in many types of human cancers, including particularly pancreatic cancer (70–90%), lung tumors (35%), colorectal carcinoma (40%) and endometrial carcinoma (78%). Mutations in k-ras occur early in disease progression and are found in great majority of invasive carcinomas. However, compared with Ha-Ras, understanding the contribution of K-Ras toward human carcinogenesis remains obscure.

Abbreviations: 5′-azaC, 5-aza-2′-deoxycytidine; DNMT, DNA methyltransferase; EDTA, ethylenediaminetetraacetic acid; HDAC, histone deacetylase; mRNA, messenger RNA; MSP, methylation-specific PCR; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA.

DNA methylation plays a major role in regulating various cellular and developmental processes (4–7). DNA methyltransferases (DNMTs) are the enzymes catalyzing DNA methylation at the 5-position (C5) of the cytosine ring in the context of CpG dinucleotides (8). DNMT1 is essential for maintaining DNA methylation patterns in proliferating cells, whereas DNMT3a and DNMT3b are de novo methyltransferases creating novel methylation patterns during embryonic development and maintaining the global methylation patterns in some cancers (9,10). However, alterations in the patterns of DNA methylation are among the earliest and most common events in tumorigenesis, leading to epigenetic silencing of the expression of tumor suppressor genes (11–16). Despite the important role of DNMTs in carcinogenesis, the molecular mechanism of DNA methylation in tumor remains largely undefined (17).

E-cadherin is a transmembrane glycoprotein that mediates Ca2+-dependent intercellular adhesion (18). E-cadherin is expressed predominantly on the surface of epithelial cells, where it plays a major role in controlling morphogenesis (19,20). E-cadherin gene is often termed a metastasis suppressor because of its suppressing effect on tumor cell invasion and metastasis. Previously, it was reported that E-cadherin gene could be hypermethylated in leukemia cells (21) while its inactivation occurred in undifferentiated solid tumors by both genetic and epigenetic mechanisms (22,23). Hypermethylation within E-cadherin promoter region was observed in gastric carcinomas (23). On the other hand, involvement of E-cadherin-based adhesion junction formation essential for epithelial tissue organogenesis was found to be induced by K-Ras, but not by Ha-Ras and N-Ras (24).

Despite much efforts, however, for unraveling the molecular mechanisms of K-Ras-induced tumorigenesis (25,26), regulation of E-cadherin expression in association with K-Ras-induced DNA methylation and cell malignancy has not been defined. In this study, we demonstrate that K-Ras can induce DNMT3b binding to E-cadherin promoter region, resulting in promoter hypermethylation and reduced expression of E-cadherin and decreased aggregation and metastatic mobilization of human prostate epithelial cancer cells overexpressing K-Ras. Furthermore, posttranslational modification of DNMT3b seems to be the reason for its increased level in the k-ras-transformed cells.

Materials and methods

Chemicals and antibodies

5-aza-2′-deoxycytidine (5′-AzaC) was purchased from Sigma Chemical Co. (St Louis, MO). Antibodies to DNMT1, GAPDH and histone deacetylase (HDAC) 1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to K-Ras (Ab-1) was obtained from Calbiochem (San Diego, CA). Antibody to DNMT3a was purchased from IMGENEX (San Diego, CA). Antibody to anti-DNMT3b was purchased from Abcam (Cambridge, UK). Matrigel and antibody to anti-E-cadherin were purchased from BD Biosciences (Erembodegem, Belgium).

Cell culture, transfection and luciferase reporter gene assay

Human neonatal prostate cell line, 267B1, was established by transfecting primary human neonatal prostate epithelial cells with a plasmid containing SV40 early region genes. These cells became immortalized but retained the essential characteristics of primary human prostate epithelial cells: an epithelial morphology, expression of cytokeratins specific for epithelial cells, an antigenic profile similar to adult prostatic epithelial cells and non-tumorigenicity in nude mice. The 267B1 cells were infected with Kirsten murine sarcoma virus containing an activated k-ras (V12) oncogene, subcultured every 7–10 days, and the morphology, growth pattern, p21 expression and tumorigenicity study in nude mice were observed for selection of transformed 267B1/K-Ras cell line (27). Maintenance and cultivation of both 267B1 and 267B1/K-Ras cell lines were described previously (28). Briefly, 267B1 and 267B1/K-Ras cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.5 μg/ml hydrocortisone and 10% heat-inactivated fetal bovine serum and
were cultured in a humidified CO₂ incubator at 37°C. For luciferase reporter assays (Promega, Madison, WI), cells (2 × 10⁵/ml) were incubated in six-well plates for 24 h and were then co-transfected with 0.5 µg of pEcad-Luc (harboring E-cadherin promoter and luciferase gene) and 0.05 µg of pCMV/β-galactosidase plasmid. In some cases, cells were treated with 5'-AzA-C after 24 h, and luciferase measurement was performed with a detection kit. The pE-cad-Luc reporter vector was kindly provided from Dr K.L.J. (Pusan National University) (29). The full-length E-cadherin promoter (GenBank accession no. AY341818) was obtained from genomic DNA of HepG2 cells by polymerase chain reaction (PCR) amplification using a primer set, Ecad-3R (5'-GCCCTGATCAGGAAATCTGGA-3') and anti-sense primer 5'-TCGTGGATCTCCCAATGTTCAAC-3' for E-cadherin, primer 5'-GGTTGTTTTCTCCAAAATACTGTA-3' and anti-sense primer 5'-TTGAGCATCTAAAAGGAGCGTGGTA-3' for DNMT1, sense primer 5'-CCTCTGCTGCTTTTGCCCTGTTGGG-3' and anti-sense primer 5'-GTCGACTCCCGCATGCTGTTGGG-3' and reverse primer 5'-CTCGAGGTCTGGTCTGCGTT-3' for reverse primer 5'-CTCGAGGCTTCCTCCCTCTTTCTTCATGG-3' and for glycosaldehyde 3-phosphate dehydrogenase, sense primer 5'-ACCGAAATCTTCCATCAGTCTGTT-3' and for anti-sense primer 5'-TACAGCCACGGGATGTTGTT-3' and anti-sense primer 5'-CACAAGGGTTATGTT-3' for luciferase. The PCR amplification was carried out using an ABI automated sequencer 3100 with Dye terminators tide triphosphate mixture and 50 pmol sense and antisense primers in a volume of 25 µl. The PCR products were cloned to pGEM easy vector and white colonies were selected for plasmid minipreparation. Positive clones were sequenced on an ABI automated sequencer 3100 with Dye terminators and Yara et al. (2008) has described the complete methylation status of CpG islands in the E-cadherin promoter: forward primer, 5’-CTCGAGGCTTCCTCCCTCTTTCTTCATGG-3’ and reverse primer 5’-TTGAGCATCTAAAAGGAGCGTGGTA-3’ for unmethylated DNA amplification, forward primer 5’-TAATTTTATGTGGTATTGGTGTT-3’ and reverse primer 5’-CACAACCAATCACAACAAAAC-3’ (19). MSP analysis of the transfected E-cadherin promoter was described previously (29).

**Chromatin immunoprecipitation analysis**

Cells were cross-linked with 1% formaldehyde at 37°C for 10 min. Cells were washed twice with ice cold phosphate-buffered saline containing protease inhibitors, scraped and pelleted by centrifugation at 4°C. Cells were resuspended in SDS lysis buffer [1% SDS, 10 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM Tris–HCl (pH 8.1)] for 10 min on ice and sonicated to shear DNA. The lysate was centrifuged at 37500 rpm, 4°C for 10 min. The supernatant was diluted in a dilution buffer [0.01% SDS, 1% Triton X-100, 1 mM EDTA, 16.7 mM NaCl, 16.7 mM Tris–HCl (pH 8.1) and protease inhibitors]. DNMT1, DNMT3a, DNMT3b and HDAc1 antibodies were added to the supernatant and incubated overnight on a rotating platform at 4°C. The immune complexes were collected with Protein-A sepharose beads and washed sequentially with low-salt washing buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris–HCl (pH 8.1) and 150 mM NaCl], high-salt buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris–HCl (pH 8.1) and 500 mM NaCl], lithium washing buffer [0.25 M LiCl, 1% Triton X-100, 1% deoxycholic acid, 1 mM EDTA and 10 mM Tris–HCl (pH 8.1)] and TE buffer [1 mM EDTA and 10 mM Tris–HCl (pH 8.1)]. The immunocomplex was eluted by divul mumol NaHCO₃, and 200 mM NaCl] and the cross-links were reversed by heating at 65°C for 4 h. The samples were adjusted to pH 6.5 with 10 mM Tris–HCl and mixed with 40 µg/ml proteinase K and further incubated at 45°C for 1 h. The E-cadherin primers are 5’-AGAGGTACCAAGAAGCTTTGATTGTT-3’ and 5’-CCTAGAGGGCAGGCTATGG-3’ for forward and reverse primers, respectively. Cell aggregation and migration through matrigel assays

Cell migration ability was evaluated by using the chemotaxis chamber as described previously (30). Briefly, matrigel was polymerized with medium containing 1 µg/ml collagen type 1 (Sigma, St. Louis, Mo) in polycarbonate transwell inserts for 1 h at 37°C. Cells (1 × 10⁵) in 1.5 ml of culture medium were applied to the upper chamber of the device, and 2.6 ml of medium was added to the lower chamber. As a control, a parallel experiment was performed using the device with uncoated membrane. After 24 h of incubation at 37°C, the membrane was fixed in methanol for 10 min and stained with Giemsa solution for 1 h. Migrated cells on the membrane were counted under a microscope. Fast aggregation assay was performed as described previously (31). Cells were dissociated with Hank’s balanced salt solution with 0.01% trypsin and 1 mM CaCl₂ and washed twice in Ca²⁺/Mg²⁺-free Hank’s balanced salt solution. The resulting cells (2 × 10⁵) were resuspended in 2 ml of Hank’s balanced salt solution containing 1 mM CaCl₂ were incubated for the indicated time at 37°C on a gyratory shaker. After incubation, the total particle number (single cells plus cell clusters) in each cell suspension was counted.

**Transfection with siRNA**

k-ras small interfering RNA (siRNA) (sc-35731) was purchased from Santa Cruz Biotechnology. Cells were transfected with 100 nM of siRNA using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) in OPTI-MEMI-reduced serum medium (Invitrogen) for 6 h. The medium was removed and replaced with fresh Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Cells were harvested 72 h after transfection for western blot analysis.

**Confocal microscopy**

Approximately 5 × 10⁶ cells grown on a glass coverslip were washed twice with phosphate-buffered saline followed by 4% paraformaldehyde fixation for 10 min at room temperature, cells were then immunolabeled using specific antibodies against E-cadherin. Cells were incubated with fluorescin isothiocyanate conjugated secondary antibody for 1 h at room temperature. Cells were mounted using Vectashield and observed on an LSM 510 META confocal microscope using a ×100 1.4 NA objective.

**Results**

K-Ras represses E-cadherin expression

We have performed microarray analysis searching for the genes downregulated by K-Ras overexpression but restored upon 5'-AzA-C treatment. Among 32 genes identified (supplementary 3 is available at Carcinogenesis Online), E-cadherin was noticeable in its expression in 267B1/K-Ras cells overexpressing K-Ras. Real-time PCR analysis showed that E-cadherin messenger RNA (mRNA) expression was significantly reduced in the transformed 267B1/K-Ras compared with
267B1 parental cells. However, E-cadherin mRNA was found to be restored by 5′-AzaC treatment (Figure 1A). These results were further confirmed when reverse transcription–PCR analysis for E-cadherin was performed in the presence or absence of 5′-AzaC for various times. Although E-cadherin mRNA was evident in 267B1 cells, complete downregulation of its expression occurred in 267B1/K-Ras cells. However, 5′-AzaC time-dependently increased E-cadherin mRNA expression (Figure 1B). Similarly, E-cadherin protein expression could also be restored by 5′-AzaC, although not so strong as its mRNA level (Figure 1C). To further support that K-Ras reduces E-cadherin expression through DNA hypermethylation, the normal 267B1 cells were transiently transfected with k-ras oncogene in the presence or absence of 5′-AzaC. It was found that increasing the amount of k-ras dose-dependently reduced the E-cadherin mRNA expression. Pretreatment with 5′-AzaC, however, significantly restored E-cadherin expression (Figure 1D). Similar pattern could also be obtained by western blot analysis (Figure 1E). These results suggest that E-cadherin expression could be suppressed by K-Ras through hypermethylation within the E-cadherin promoter region.

**K-Ras represses E-cadherin expression by inducing hypermethylation of E-cadherin promoter**

Since expression of E-cadherin in K-Ras overexpressing cells was restored by 5′-AzaC (Figure 1), the methylation status of the promoter region of E-cadherin was examined in detail. Similar to other numerous studies demonstrating silencing of the E-cadherin expression in many tumor types, typical CpG islands were found on the E-cadherin promoter region as well as the first exon (Figure 2A), reflecting a recent suggestion that aberrant DNA methylation of CpG islands around promoter regions could inactivate tumor suppressor genes with a similar potency to the mutations in the coding regions (31). Thus, a reporter gene analysis was performed in 267B1 cells co-transfected with pEcad-luc and a plasmid harboring k-ras. Consistent with the above results (Figure 1D and E), K-Ras overexpression significantly reduced E-cadherin promoter activity, whereas 5′-AzaC treatment restored it to some extent (Figure 2B). Similarly, 5′-AzaC increased E-cadherin promoter activity in 267B1/K-Ras cells at all time points, although there appeared some cytotoxicity by longer exposure (Figure 2C).

Next, to further confirm that decreased expression of E-cadherin is associated with promoter hypermethylation, the methylation status in CpG island (−177 to −63 from the transcription start site) of E-cadherin promoter was determined by MSP. Methylation of E-cadherin promoter could only be detected in 267B1/K-Ras, whereas unmethylated form was only observed in normal 267B1 cells (Figure 2D). Treatment of 5′-AzaC to the cells time-dependently increased demethylation of the E-cadherin promoter region. A similar result could be obtained when 267B1 cells were transiently transfected with a vector harboring k-ras oncogene. Decreasing the amount of unmethylated form by K-Ras expression was accompanied by the appearance of the methylated form, complete shift from methylated to unmethylated E-cadherin promoter being obtained by 5′-AzaC treatment (Figure 2E).

Based on the above data, bisulfite sequencing analysis was conducted for the evaluation of DNA methylation status within the 5′ regulatory region of E-cadherin. Bisulfite treatment converts cytosines into uracils, leaving methylated cytosines unaffected. In examining the methylation status of 37 CpG dinucleotides (−333 to +153 bp) using...
two sets of primers, PCR products were cloned into pGEMT-easy vector and after transformation, 10 independent white colonies were examined for each of the three cells, 267B1, 267B1/K-Ras and 267B1/K-ras treated with 5′-azaC. The methylated cytosine residues in each clone were represented by a solid spot (Figure 3A). Compared with 267B1 cells, methylation of 22 CpG sites predominantly occurred in E-cadherin promoter region (−214 to −103 bp) and part of exon 1 (36–116 bp), but among them, only 4 CpG sites were significantly changed by 5′-azaC in 267B1/K-Ras cells (−131 to −103 bp), raising the possibility that promoter hypermethylation regulates E-cadherin expression by K-Ras.

Increased binding of DNMT3b to E-cadherin promoter in 267B1/K-ras cells

Given the observations demonstrating that E-cadherin promoter methylation was increased by K-Ras expression (Figure 3A), DNMT enzymes were expected to be upregulated in expression and directly bind to the promoter region of E-cadherin in 267B1/K-Ras cells. Unexpectedly, however, DNMT3b was significantly expressed in the transformed cells, whereas the amounts of DNMT1 and DNMT3a were the same in both 267B1 and 267B1/K-ras cells (Figure 3B). Moreover, k-ras knockdown by siRNA treatment only downregulated DNMT3b expression.

DNA methylation is mediated by the recruitment of protein factors including DNMTs and transcription repressors such as methyl-binding proteins, which are part of a large complex that includes HDACs, essential components in methylation-dependent transcriptional repression. To investigate whether induction of DNA methylation and transcriptional repression complex formation can occur on E-cadherin promoter, chromatin immunoprecipitation analysis was performed by immunoprecipitating formaldehyde cross-linked protein–chromatin complexes using antibodies against DNMT1, DNMT3a, DNMT3b and HDAC1. In agreement with the protein expression patterns, DNMT3b was found to bind to the E-cadherin promoter region in 267B1/K-Ras cells (Figure 3C). Furthermore, 5′-azaC treatment blocked DNMT3b binding without having any effect on DNMT1 and DNMT3a. In addition, HDAC1 binding to the promoter was increased in 267B1/K-Ras cells but did not change with the treatment of 5′-azaC. Thus, our results suggest that K-Ras induce DNA methylation and formation of a transcriptional repression complex.
repression complex containing DNMT3b and HDAC1 within E-cadherin promoter, leading to the transcriptional repression and silencing of E-cadherin.

**K-Ras upregulates DNMT3b protein expression**

Since DNMT3b was revealed to be highly expressed and to bind to E-cadherin promoter but dissociate upon 5'-AzaC treatment (Figure 3C), it was expected that 5'-AzaC could reduce DNMT3b expression. As expected, the amount of DNMT1 and DNMT3b mRNAs did not change regardless of 5'-AzaC (Figure 4A). Interestingly, however, DNMT3b transcriptional level was also the same in both 267B1 and 267B1/K-Ras cells (Figure 4A), although DNMT3b protein expression was higher in the transformed cells (Figures 3B and 4B). Furthermore, 5'-AzaC treatment induced the degradation of DNMT1 without effect on DNMT3b (Figure 4B). These results were confirmed by transfecting 267B1 normal cells with a plasmid harboring k-ras in the presence or absence of 5'-AzaC (Figure 4C and D). K-Ras or 5'-AzaC could not affect the transcriptional expression of either DNMT1 or DNMT3b (Figure 4C). Western blot analysis, however, showed that DNMT3b was only increased by k-ras transfection and that 5'-AzaC induced the degradation of DNMT1 without effect on DNMT3b (Figure 4D). These results suggest a post-modification mechanism for the regulation of DNMT3b expression. In addition, degradation of DNMT1 and dissociation of DNMT3b upon 5'-AzaC treatment to 267B1/K-Ras cells could be associated with the E-cadherin expression.

**K-Ras-mediated loss of E-cadherin expression affects cell–cell interaction and migration of the transformed cells**

It has previously been shown that loss of E-cadherin expression resulted in morphological changes and enhanced cell migration activity, which significantly correlated with tumor metastasis. Therefore, we examined in vitro migration assay to confirm the correlation between invasive migration and reduction of E-cadherin expression. Both 267B1 and 267B1/K-Ras cells were either treated with 5'-AzaC or left untreated. Examining cell aggregation ability of the two cell
types, most of 267B1 cells aggregated well and showed little change regardless of 5'-AzaC treatment. However, 267B1/K-Ras cells were dispersed but aggregation could be restored upon 5'-AzaC treatment (Figure 5A, upper). In agreement with the enhanced cell aggregation, E-cadherin expression was also increased in 267B1/K-Ras cells treated with 5'-AzaC while it was highly expressed in 267B1 cells regardless of 5'-AzaC treatment (Figure 5A, middle). In consistent with the reduced cell aggregation, the number of 267B1/K-Ras cells that migrated through the membrane pore was significantly higher than that of the normal cells but was reduced to the normal level after treatment with 5'-AzaC (Figure 5A, lower, and Figure 5B), indicating that K-Ras-induced E-cadherin repression increases cell invasive potential. Taken together, our results suggest that loss of E-cadherin is responsible for the morphological changes, altered cell–cell interactions and eventually cell metastasis induced by K-Ras.

Discussion

Disruption of E-cadherin in many different cancer cells has been reported (32–34). The molecular mechanism that regulates silencing of E-cadherin in cells overexpressing K-Ras (supplementary 1 is available at Carcinogenesis Online), however, is not well elucidated. Following a microarray analysis (supplementary 3 is available at Carcinogenesis Online) and repeated real-time PCR, E-cadherin was found to be downregulated but was restored by 5'-AzaC treatment. Exploitation of E-cadherin promoter showed that four specific CpG sites (−131 to −103 bp) were induced to be hypermethylated by K-Ras and could be demethylated by 5'-AzaC treatment (Figure 3A). Comparing the methylation sites from a previous report demonstrating several CpG sites methylation (−260 to −10) in latent membrane protein 1-induced MCF7 breast cancer cells (35); however, it was found that most of the methylation sites occurring in this breast cancer cell type differ from those observed in 267B1/K-Ras cells. In our study, the four CpG sites regulated by 5'-AzaC are located near to E-boxes and other binding sites for some transcription factors including stimulatory protein -1. Moreover, this region encompassing the four CpG sites was shown to be crucial for E-cadherin expression when measured by luciferase reporter gene assay using deletion mutants of E-cadherin promoter in MDA231 and MCF7 cells (36). Thus, a possibility could be that proximity of DNA methylation sites near the transcription factor binding sites would be required for efficient transcriptional regulation of E-cadherin as well as other tumor suppressor genes. However, in order to support this suggestion, further study is required for unraveling the molecular interaction of E-cadherin promoter methylation and transcription factor binding.

A previous report demonstrated that Trichostatin A, a potent inhibitor of HDAC, decreased DNMT3b expression in human endometrial cells (37). In our study, DNMT3b and HDAC1 could bind to the promoter region, whereas DNMT3b only dissociated from the CpG sites upon 5'-AzaC treatment (Figure 3C). Since DNA methylation and histone deacetylation are closely associated with gene expression, our data suggest that E-cadherin expression could be regulated by both mechanisms and that HDAC family members could play a pivotal role in K-Ras-induced gene expression. In support of this, Trichostatin A affected the migration of 267B1/K-Ras cells while showing no effect on 267B1 cells (supplementary 4 is available at Carcinogenesis Online). In this respect, the involvement of other HDAC proteins in E-cadherin regulation needs to be further resolved in association with DNA methylation. Thus, given that hypermethylation of the promoter is critical for reducing cadherin expression during the metastasis of carcinoma (38), the specific region of E-cadherin promoter (−131 to −103 bp) seems to be important for K-Ras-induced cell metastasis through both DNA methylation and histone acetylation.

DNMT1 is responsible for maintenance DNA methylation, whereas DNMT3a is responsible for de novo methylation. In our study, high level of K-Ras or knockdown of k-ras gene in 267B1/K-Ras cells did not affect the amount of the two DNMT enzymes (Figure 3B), and there was no change in their association with the E-cadherin promoter.
even in K-Ras-overexpressing stable cell line with or without 5′-AzaC treatment (Figure 3C). Moreover, mRNA level of DNMT1 and DNMT3b remained unchanged regardless of K-Ras expression or 5′-AzaC treatment (Figure 4A and C). Western blot analyses, however, showed that DNMT3b level was increased by K-Ras expression (Figure 4B and D). These results suggest that expression of DNMT3b might be regulated by posttranslational modification, whereas DNMT3a might have little effect on E-cadherin expression in K-Ras overexpressing cells. On the other hand, the observation that 5′-AzaC lowered the amount of DNMT1 in K-Ras expressing cells (Figure 4B) reflects a supporting evidence that proteolytic degradation of DNMT1 after 5′-AzaC treatment was observed in cancer cells (supplementary 2 is available at Carcinogenesis Online, 39). However, detailed elucidation of the regulatory mechanism of the DNMT1 degradation and of the increase in translational expression of DNMT3b by K-Ras remains to be elucidated.

The association of RAF and DNA methylation has been exploited (40,41). Activation of the RAS/Ras-activated factor/mitogen-activated protein kinase intracellular signaling pathways has been shown to induce DNMTs, and the elevated activity of DNMTs levels are required to maintain the phenotype of Ras-transformed cells (42,43). We previously showed that K-Ras could activate extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase intracellular signaling pathways (25). Given that Hepatitis B Virus X induced DNMT1 activation (29), whereas K-Ras increased DNMT3b binding to E-cadherin promoter without effect on DNMT1 in our study, exploitation of the interaction of DNMT3b expression and mitogen-activated protein kinase activation would be promising for the treatment of K-Ras-induced tumorigenesis. Our study showed that cell surface expression of E-cadherin and the subsequent cell–cell interaction and migration were regulated by 5′-AzaC treatment (Figure 5). However, 5′-AzaC could also affect other signaling factors. In this respect, a subset of genes significantly downregulated in tumor-conditioned endothelial cells was reactivated by treatment of 5′-AzaC or Trichostatin A without association with DNA methylation of promoter CpG islands (44). Thus, it remains to be elucidated whether E-cadherin in association with DNA methylation directly affects the tumorigenic characteristics of the K-Ras transformed cells or requires other molecules such as those regulated by histone acetylation.

In summary, reduced expression of E-cadherin by hypermethylation of four CpG sites in the promoter in 267B1/K-Ras cells could be associated with cell aggregation and metastatic movement, and DNMT3b might be responsible for the hypermethylation of E-cadherin promoter. Although there has been controversies regarding the role of DNMT expression in tumorigenesis showing that DNMT
expression was not closely related with DNA methylation and that genome-wide hypomethylation could occur in tumor cells (45,46), our study suggests that E-cadherin, regulated by DNMT3b binding on its promoter, could function as a tumor suppressor in K-Ras expressing prostate cancer cells.

Supplementary material

Supplementary Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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